Pathogenicity and Transmissibility of a Novel H3N3 Subtype Avian Influenza Virus in Chickens

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Abstract

Novel avian influenza viruses can cause severe economic losses to the poultry industry, making timely detection and interruption of their transmission critically important. During 2022-2023, our laboratory monitored the emergence and spread of a novel H3N3 subtype avian influenza virus in diseased chicken flocks in East and North China. This study investigated the clinical manifestations in affected flocks, performed whole-genome evolutionary analysis of isolated viruses, and conducted pathogenicity and airborne transmission experiments in chickens using representative isolates. The results demonstrated: 1) H3N3 first emerged in December 2022 in laying hen flocks with decreased egg production in East China, and was subsequently detected in chicken flocks across multiple regions including East China, North China, and Northeast China; the affected population was primarily laying hens, with high flock morbidity but low mortality (1%-5%), mainly causing acute declines in egg production (10%-40%); 2) Whole-genome sequence analysis revealed that the H3N3 virus is a novel reassortant virus, generated through reassortment between the H3N8 virus circulating in chicken flocks and the H10N3 virus, with six internal genes derived from the H9N2 virus; 3) Similar to the H3N8 virus, the novel H3N3 virus shows high infectivity for chickens, can replicate efficiently and shed virus in the chicken respiratory system, causing severe pathological damage to the respiratory tract; 4) Airborne transmission experiments demonstrated that the H3N8 virus cannot be effectively transmitted through the air between chickens, whereas the novel H3N3 virus can be effectively transmitted through the air between chickens. In summary, the novel H3N3 avian influenza virus exhibits strong pathogenicity and transmissibility in chickens, may become a new dominant epidemic strain, poses a significant threat to China' s poultry industry, and necessitates research on its prevention and control.

Full Text

Pathogenicity and Airborne Transmissibility of Novel H3N3 Subtype Avian Influenza Virus in Chickens

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Abstract

Novel avian influenza viruses (AIVs) can cause serious economic losses to the poultry industry. Systematic surveillance of novel AIVs in poultry is essential for control of avian influenza. In the present research, we isolated H3N3 subtype AIVs from chicken farms reporting illness in China during 2022–2023. All of these chickens showed respiratory disease signs and a 10-40% reduction in egg production. We conducted epidemiological surveys, virus isolation and identification, sequence analysis, and chicken infection experiments. The results showed that: 1) In December 2022, H3N3 AIVs were first found in a layer chicken flock in Eastern China, and the virus was transmitted to multiple provinces with high density chicken populations in a short time. 2) Sequence analysis showed that the novel H3N3 AIVs were evolving as a triple reassortment event, which bears an H3N8-derived HA gene, an H10N3-derived NA gene, and H9N2-derived internal genes. 3) The novel H3N3 AIVs were highly susceptible to SPF chickens. The virus replicated efficiently in the concha nasalis, trachea, lungs, and Harders glands, and infected chickens showed pathological damage. 4) H3N3 viruses were airborne transmissible among chickens, whereas H3N8 viruses were not. In conclusion, the novel reassortment H3N3 virus showed pathogenicity and airborne transmissibility in chickens. Therefore, comprehensive surveillance of H3N3 AIVs in domestic poultry is imperative, and control of the virus endemic is needed.

Keywords: H3N3; Avian Influenza Virus; Chicken; Pathogenicity; Airborne Transmissibility

Introduction

Avian influenza virus (AIV) belongs to the Orthomyxoviridae family, genus Influenza A virus, with a genome composed of eight single-stranded negative-sense RNA segments. When two influenza viruses co-infect the same host cell, exchange of viral gene segments may occur, also known as genetic reassortment,

resulting in new subtype or genotype viruses. Novel influenza viruses can pose serious threats to the poultry industry or human health; therefore, timely detection of novel influenza viruses is of great significance.

The H9N2 subtype AIV is the most common virus in chicken flocks, with multiple evolutionary lineages, among which the BJ/94 lineage H9N2 virus is the predominant circulating lineage in Chinese chicken flocks [**?**, **?**]. After 2010, the G57 genotype of the BJ/94 lineage H9N2 virus became dominant in Chinese chicken flocks [**?**]. This genotype not only exhibits enhanced pathogenicity to chickens but, more importantly, can serve as a gene donor providing internal genes for other subtype influenza viruses, generating various novel viruses such as H7N9, H10N3, H10N8, and H5N6 [**?**]. In 2022, a novel H3N8 AIV emerged in Chinese chicken flocks, which was formed through triple reassortment involving an H3 gene from waterfowl-origin viruses, an N8 gene from North American wild bird-origin viruses, and internal genes from H9N2 viruses [**?**]. This novel virus can replicate at high levels in the chicken respiratory system, causing severe pathological damage, and due to the general lack of specific antibodies against H3 subtype AIV in chicken flocks, this novel virus rapidly spread and became prevalent in Chinese chicken flocks [**?**], causing substantial economic losses to the poultry industry. The co-circulation of different subtypes of low pathogenicity AIV in Chinese chicken flocks may lead to further genetic reassortment and generation of novel viruses; therefore, conducting etiological surveillance of AIV in Chinese chicken flocks is a critical component of scientific avian influenza prevention and control.

For many years, our laboratory has closely monitored the emergence and evolutionary dynamics of novel influenza viruses. Clinical investigations revealed that since December 2022, a novel H3N3 subtype AIV has been detected in chicken flocks with decreased egg production in Eastern, Northern, and Northeastern China. Whole-genome sequence analysis showed that this virus was generated through reassortment between H3N8 and H10N3 viruses. To elucidate the pathogenic and transmissibility characteristics of the novel H3N3 virus in chickens, this study conducted chicken infection experiments with isolated strains in SPF chickens and evaluated airborne transmissibility among SPF chickens, aiming to provide a theoretical basis for scientific prevention and control of this disease.

Materials and Methods

1.1.1 Sample Collection and Processing

During 2022-2023, we conducted AIV surveillance on poultry farms in major poultry-producing provinces of China. Oropharyngeal swabs were collected from diseased chickens showing typical influenza clinical features such as respiratory symptoms and decreased egg production. For dead chickens, tracheal and lung tissue samples were collected during necropsy. All samples were placed in centrifuge tubes containing 1 mL sterile PBS (with antibiotics) and transported

under low temperature to the Key Laboratory of Avian Influenza and Other Major Poultry Diseases Prevention and Control, Ministry of Agriculture and Rural Affairs, China Agricultural University for virus isolation and identification.

1.1.2 Pathogen Nucleic Acid Identification

Two hundred microliters of resuspended oropharyngeal swab supernatant or tissue homogenate were used for RNA extraction using an RNA extraction kit. Real-time fluorescent quantitative RT-PCR was performed to detect avian influenza virus, Newcastle disease virus (NDV), and infectious bronchitis virus (IBV).

1.1.3 Avian Influenza Virus Isolation and Identification

Samples positive for influenza virus nucleic acid by real-time fluorescent quantitative RT-PCR were inoculated into 9-day-old SPF chicken embryos and incubated at 35°C for 48 hours. Allantoic fluid was harvested after overnight refrigeration at 4°C. Hemagglutination (HA) and hemagglutination inhibition (HI) assays were performed to determine the HA subtype of the influenza viruses. RNA was extracted from the allantoic fluid and reverse-transcribed into cDNA using universal influenza virus primers unit12. Whole-genome sequencing of the virus was performed using Hoffmann primers [**?**].

1.1.4 Genetic Evolution Analysis

Whole-genome sequences of the isolates were subjected to BLAST sequence alignment in the GISAID database. Reference sequences for each gene segment were selected, and maximum-likelihood phylogenetic trees were constructed using FastTree 2.1.11 software. Branch support values were calculated using the Shimodaira-Hasegawa (SH) method with 1,000 bootstrap replicates.

1.2.1 Experimental Design

SPF Chicken Pathogenicity Experiment: Eight 4-week-old SPF chickens per group were intranasally inoculated with virus at a dose of 10^6 EID50. Clinical signs were observed daily. Three inoculated chickens were necropsied at 4 days post-infection (dpi) to observe pathological changes, and organs were collected for histopathological examination and virus titration. Oropharyngeal and cloacal swabs were collected daily from infected chickens from 1 to 14 dpi for virus titration. Blood was collected on day 14 to detect seroconversion by HI assay.

SPF Chicken Airborne Transmissibility Experiment: Ten 4-week-old SPF chickens per group were used. Five chickens were intranasally inoculated with virus at a dose of 10^6 EID50. At 24 hours post-inoculation, these five chickens were placed in an adjacent cage, with a distance of 30 cm between

cages. Airflow was directed from the inoculated group toward the airborne contact group. Clinical signs were observed daily. Oropharyngeal and cloacal swabs were collected daily from both inoculated and airborne contact groups from 1 to 16 dpi for virus titration. Blood was collected on day 21 to detect seroconversion by HI assay.

1.2.2 Histopathological Sections and Immunohistochemistry

Turbinate, tracheal, lung, and other tissues were fixed in 4% paraformaldehyde solution, processed into paraffin sections, and subjected to HE staining and immunohistochemical staining. The primary antibody for immunohistochemistry was influenza A virus NP antibody.

1.2.3 Virus Load Determination

Oropharyngeal and cloacal swabs were added to 1 mL sterile PBS containing antibiotics, vortexed, and centrifuged at low speed. The supernatant was collected and serially diluted in 10-fold increments. Turbinate, trachea, lung, liver, spleen, kidney, brain, duodenum, thymus, bursa of Fabricius, and Harderian gland tissues were collected. Sterile PBS containing antibiotics was added at a ratio of 0.1 g/mL, and tissues were thoroughly homogenized using a tissue homogenizer. After low-speed centrifugation, the supernatant was serially diluted in 10-fold increments. Samples were inoculated into 10-day-old SPF chicken embryos at 0.2 mL per embryo, with three embryos per dilution. After inoculation, embryos were incubated at 35°C for 48 hours, then refrigerated at 4°C overnight. Allantoic fluid was harvested to determine hemagglutination titers, and virus titers were calculated using the Reed-Muench method.

Results

2.1 Clinical Disease and Virus Prevalence

Since December 2022, an infectious disease characterized by respiratory symptoms and acute egg production decline has occurred successively in laying chicken flocks, including commercial layers, broiler breeders, and layer breeders, in Eastern, Northern, and Northeastern China. The disease spread rapidly, with chickens in different houses of the same farm showing clinical signs within one week. Affected flocks exhibited high morbidity but low mortality (approximately 1-5%), with the primary manifestation being acute decline in egg production. Typical clinical signs in diseased chickens included swollen conjunctiva, open-mouth breathing with respiratory rales, and a sharp 10-40% drop in egg production within one week, along with poor eggshell quality. Necropsy findings revealed swollen and hemorrhagic eyelids, abundant purulent secretions in the nasal cavity, hyperemic oral mucosa, large amounts of mucus in the choana, diffuse hemorrhage in the trachea (Fig. 1 [FIGURE:1]a), substantial mucus adhesion to the mucosal layer (Fig. 1b), pulmonary congestion and consolidation (Fig. 1c), yellow fibrinous exudate blocking bronchioles

(Fig. 1d), follicular congestion, hemorrhage, liquefaction, and rupture leading to yolk peritonitis (Fig. 1e,f), and hyperemic oviduct mucosa with secretory exudate. The disease course lasted over one month, with slow recovery of egg production. The mortality and culling rate in the later stage was closely related to management practices and control of secondary infections.

Fig. 1 Typical clinical and pathological changes of diseased chickens

2.2 Isolation, Identification, and Genetic Evolution Analysis of Novel H3N3 Virus

The H3N3 subtype virus was first detected in December 2022 in commercial layer flocks with decreased egg production in Eastern China and subsequently detected in diseased layer flocks in multiple regions including Northern and Northeastern China. Representative strains were selected for whole-genome sequencing based on virus isolation time and location. Phylogenetic analysis revealed that the HA gene of H3N3 viruses belonged to the Eurasian avian lineage, clustering in the same evolutionary branch as the H3N8 viruses circulating in Chinese chicken flocks in 2022, with genetic similarity of 96.9%-99.3% to H3N8 viruses in the same branch (Fig. 2 [FIGURE:2]). The NA gene also belonged to the Eurasian avian lineage, clustering in the same evolutionary branch as the H10N3 viruses circulating in chicken flocks in Eastern China, with genetic similarity of 96.9%-99.0% to H10N3 viruses in the same branch (Fig. 2). The six internal genes of the sequenced strains all originated from the G57 genotype H9N2 subtype virus prevalent in Chinese chicken flocks. The HA cleavage site of all H3N3 viruses was PEKQTR/GLF, without insertion of a polybasic cleavage site. These findings indicate that the novel H3N8 virus underwent reassortment with H10N3 virus during circulation in chicken flocks, generating the new H3N3 subtype virus.

Fig. 2 Phylogenetic tree of H3N3 AIVs

2.3 Pathogenicity of Novel H3N3 Virus in SPF Chickens

Given that the NA protein plays important roles in virus infection, budding, release, and transmission, it is necessary to evaluate changes in the pathogenicity and transmissibility of the novel H3N3 virus in poultry. This study selected two H3N3 viruses, A/chicken/Eastern China/F1225/2022 (EC/F1225) and A/chicken/Northern China/F2341/2023 (NC/F2341), for pathogenicity experiments in SPF chickens. The results showed that no chickens infected with either H3N3 virus died during the 14-day experimental period, indicating that H3N3 virus is a low pathogenicity AIV. However, chickens infected with both strains exhibited obvious clinical signs, including ruffled feathers, neck retraction, decreased feed intake, and some chickens showed eye swelling, tearing, and hemorrhagic lesions (Fig. 3 [FIGURE:3]a and b). At 4 dpi, three infected chickens from each group were necropsied, revealing swollen and hyperemic conjunctiva, hemorrhage in Harderian glands, mucus exudate in turbinates, hyperemic

tracheal mucosa with petechial hemorrhages and mucus secretions (Fig. 3c), pulmonary congestion and hemorrhage with focal consolidation (Fig. 3d), enlarged kidneys, thymic hemorrhage (Fig. 3e), and swollen and hemorrhagic cecal tonsils (Fig. 3f). Histopathological observation revealed necrosis and sloughing of conjunctival epithelial cells, vascular congestion in the submucosa, and massive inflammatory cell infiltration. In turbinates, there was sloughing of mucosal epithelial cells, lymphoid follicle hyperplasia in the submucosa, massive inflammatory cell infiltration, and capillary dilation and congestion (Fig. 4

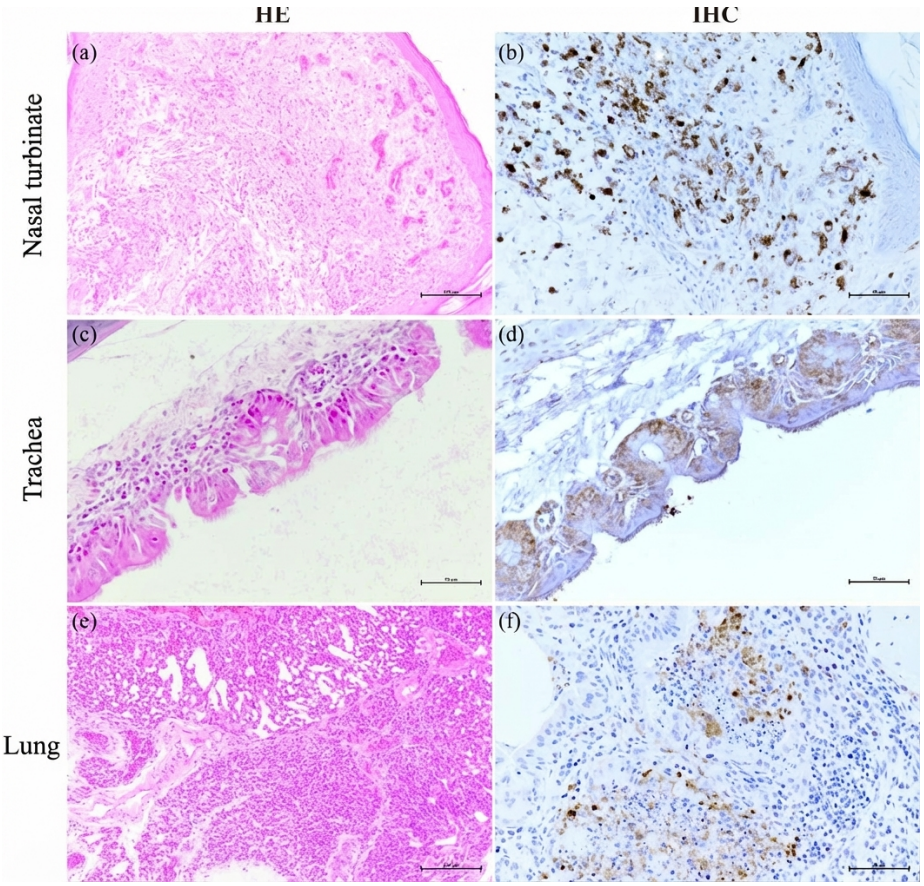

Figure 1: Figure 4

a). In the trachea, there was necrosis and sloughing of ciliated epithelial cells, inflammatory cell infiltration in the submucosa, and vascular congestion (Fig. 4c). In the lungs, there was congestion, hemorrhage, massive inflammatory cell infiltration, leading to consolidation of some pulmonary air capillaries (Fig. 4e). In situ detection by immunohistochemistry revealed abundant influenza virus antigen-positive signals in submucosal cells of turbinates (Fig. 4b), ciliated

epithelial cells of trachea (Fig. 4d), and terminal bronchiolar epithelial cells of lungs (Fig. 4f). Detection of virus distribution and load in organs showed (Fig. 5 [FIGURE:5]) that both H3N3 viruses could efficiently replicate in the chicken respiratory system, with virus titers in turbinates and trachea significantly higher than those in lungs, indicating that H3N3 viruses preferentially replicate in the upper respiratory tract of chickens. Additionally, virus could be detected in extrapulmonary organs such as Harderian glands, thymus, and brain, demonstrating that H3N3 virus has broad tissue tropism in chickens and can cause systemic infection. These results indicate that the novel H3N3 virus can replicate at high levels in the chicken respiratory system, causing severe pathological damage.

Fig. 3 Gross lesions in SPF chickens inoculated with the H3N3 AIVs

Fig. 4 Histopathological changes of SPF chickens inoculated with the H3N3 AIVs

Fig. 5 Virus titration of H3N3 AIVs in SPF chicken organs

2.4 Airborne Transmissibility of Novel H3N3 Virus Among SPF Chickens

The ability of influenza viruses to transmit via airborne routes among animal populations is a key factor for their establishment as dominant epidemic strains. This study evaluated the airborne transmissibility of H3N3 subtype AIV among chickens, using two H3N8 viruses, A/chicken/Henan/F0316/2022 (HN/F0316) and A/chicken/Anhui/FE12/2022 (AH/FE12), as controls. The results showed that virus shedding was detected in both oropharyngeal and cloacal swabs from inoculated chickens for both H3N8 viruses, with significantly higher shedding from the oropharynx than from the cloaca. The shedding period lasted up to 7 days, with peak shedding occurring at 2-5 dpi (Fig. 6 [FIGURE:6]). During the 21-day experimental period, no virus shedding or seroconversion was detected in airborne contact chickens for either H3N8 virus, indicating that H3N8 viruses cannot efficiently transmit via airborne routes among chickens. For both H3N3 viruses, virus shedding was detected in oropharyngeal and cloacal swabs from inoculated chickens, with significantly higher shedding from the oropharynx than from the cloaca. The shedding period lasted up to 7 days, with peak shedding at 2-5 dpi (Fig. 6). Notably, airborne transmission of H3N3 virus EC/F1225 began at 7 dpi, and by day 8, chickens in the airborne contact groups for both viruses showed virus shedding, which persisted for 6-8 days. HI antibody detection revealed that chickens in both airborne contact groups were HI antibody-positive. These results demonstrate that both H3N8 and H3N3 viruses are primarily shed via the oral route of chickens, but H3N3 virus possesses the ability for airborne transmission among chickens, which may facilitate its rapid spread and prevalence in chicken flocks.

Fig. 6 Replication and airborne transmission of the H3N8 and H3N3 AIVs in SPF chickens

Discussion

In this study, through disease surveillance of chicken flocks in China, we identified a novel H3N3 virus generated through reassortment between H3N8 and H10N3 viruses circulating in Chinese chicken flocks, with internal genes derived from H9N2 virus. The H3N3 virus can replicate and shed at high levels in the chicken respiratory system, causing respiratory lesions. More importantly, the H3N3 virus possesses the ability for airborne transmission among chickens, and this enhanced transmissibility may facilitate rapid spread and establishment of the virus in chicken flocks.

Genetic reassortment is an important mechanism for generating novel influenza viruses. In 2022, a novel H3N8 influenza virus was identified in diseased chicken flocks in China, which was composed of a triple reassortment involving waterfowl-origin H3Ny virus, wild bird-origin HxN8 virus, and G57 genotype H9N2 virus [**?**]. Due to carrying the six internal genes of H9N2 virus, H3N8 virus can effectively replicate and shed in the chicken respiratory system and cause clinical disease in infected chickens [**?**]. Since its emergence, H3N8 virus has rapidly spread from south to north in chicken flocks and has undergone dynamic reassortment with internal genes of H9N2 viruses circulating in different regions, generating multiple genotype viruses [**?**, **?**]. In this study, we detected that the HA gene of H3N3 virus was derived from H3N8 virus, while the NA gene originated from H10N3 virus. H10N3 virus is mainly endemic in chicken flocks in Eastern China [**?**]. We speculate that H3N8 virus co-infected with H10N3 virus in chicken hosts during circulation, resulting in reassortment and generation of the novel H3N3 virus. Given that multiple subtypes of influenza viruses, including H9N2, H10N3, H10N8, and H7N9, are circulating in Chinese chicken flocks, the probability of inter-subtype reassortment generating new genotypes and subtypes is high, necessitating close attention to emerging novel viruses.

Most H3N3 viruses detected in this study were isolated from laying hens during the egg production period, which may be related to increased susceptibility to influenza viruses due to physiological and hormonal changes in laying hens. Pathogenicity results showed that although the novel H3N3 virus could not kill SPF chickens, it caused severe pathological damage to turbinates, trachea, lungs, and conjunctiva, destroying the integrity of the respiratory mucosa. Direct tissue damage caused by H3N3 virus infection in laying hens, together with secondary infections by bacteria and mycoplasma, can trigger severe inflammatory responses and sharp increases in body temperature, leading to follicular congestion, liquefaction, and rupture, ultimately resulting in yolk peritonitis. This not only causes acute egg production decline in flocks within a short period but also results in prolonged recovery time and substantial economic losses. It should be noted that the clinical manifestations of H3N3 virus infection in chicken flocks are similar to those of low pathogenicity AIVs such as H9N2. Continuous surveillance of broilers and growing pullets showing respiratory symptoms in clinical settings is needed to understand the prevalence and pathogenicity of

H3N3 virus in different chicken breeds.

Airborne and direct contact transmission are the main transmission modes of influenza viruses. Influenza viruses primarily transmit through fecal-oral direct contact among wild waterfowl hosts, and different subtypes exhibit vastly different transmission capacities among chicken flocks. Although highly pathogenic H5Ny and H7N9 influenza viruses are highly pathogenic to chickens, they mainly transmit through direct contact and cannot efficiently transmit via airborne routes among chickens, resulting in slower spread [**?**, **?**]. Most H9N2 viruses can efficiently transmit via airborne routes among chickens, which is an important reason for their establishment as dominant prevalent strains in chicken flocks. Studies have identified that HA-K363 and PA-L672 mutations are key determinants for airborne transmission of H9N2 viruses [**?**]. In this study, we found that although both H3N3 and H3N8 viruses have reassorted internal genes from H9N2 virus and possess the PA-L672 mutation, differences in their NA genes resulted in distinct transmission characteristics. H3N3 virus could transmit via airborne routes among chickens, whereas H3N8 virus could not efficiently transmit via airborne routes among chickens. The NA protein possesses neuraminidase activity, which can cleave sialic acids on mucin and glycoprotein molecules on the surface of respiratory epithelial cells during virus infection, facilitating virus infection; during virus budding and release, it cleaves sialic acids on host cell surfaces and nascent virion surfaces, promoting virus release [**?**]. Therefore, the NA protein plays an important role in influenza virus pathogenicity, transmissibility, and cross-species transmission capacity. Compared to the wild bird-origin N8 protein, the chicken-origin N3 protein is apparently better adapted to chicken hosts. The molecular mechanisms underlying the differences in virus transmissibility caused by different subtype NA proteins require further in-depth investigation.

In summary, given the strong pathogenicity and transmissibility of the novel H3N3 virus in chickens, there is an urgent need to conduct systematic etiological and serological surveillance to understand the epidemic regions and host range of the virus, develop rapid diagnostic methods and vaccines, and formulate scientifically sound prevention and control measures to contain this novel influenza virus at the early stage of its prevalence.

Conclusion

This study discovered that the novel H3N3 influenza virus was generated through reassortment between H3N8 and H10N3 viruses circulating in chicken flocks, has formed an epidemic trend in Chinese chicken flocks, and mainly causes clinical disease in laying hens. The novel H3N3 influenza virus can effectively replicate in the chicken respiratory system, causing severe pathological damage, and the virus can efficiently transmit via airborne routes among chickens. It has the potential to become a new dominant prevalent virus in chicken flocks, posing a significant threat to China' s poultry industry, necessitating research on its prevention and control.

References

1. Sun Y, Liu J. H9N2 influenza virus in China: a cause of concern[J]. Protein Cell, 2015, 6(1):18-25
2. Gu M, Xu L, Wang X, Liu X. Current situation of H9N2 subtype avian influenza in China[J]. Vet Res, 2017, 48(1):49
3. Pu J, Wang S, Yin Y, Zhang G, Carter RA, Wang J, Xu G, Sun H, Wang M, Wen C, Wei Y, Wang D, Zhu B, Lemmon G, Jiao Y, Duan S, Wang Q, Du Q, Sun M, Bao J, Sun Y, Zhao J, Zhang H, Wu G, Liu J, Webster RG. Evolution of the H9N2 influenza genotype that facilitated the genesis of the novel H7N9 virus[J]. Proc Natl Acad Sci U S A, 2015, 112(2):548-53
4. Liu WJ, Wu Y, Bi Y, Shi W, Wang D, Shi Y, Gao GF. Emerging HxNy Influenza A Viruses[J]. Cold Spring Harb Perspect Med, 2022, 12(2):a038406
5. Yang R, Sun H, Gao F, Luo K, Huang Z, Tong Q, Song H, Han Q, Liu J, Lan Y, Qi J, Li H, Chen S, Xu M, Qiu J, Zeng G, Zhang X, Huang C, Pei R, Zhan Z, Ye B, Guo Y, Zhou Y, Ye W, Yao D, Ren M, Li B, Yang J, Wang Y, Pu J, Sun Y, Shi Y, Liu WJ, Ou X, Gao GF, Gao L, Liu J. Human infection of avian influenza A H3N8 virus and the viral origins: a descriptive study[J]. Lancet Microbe, 2022, 3(11):e824-e834
6. Sun H, Tong Q, Li H, Han Q, Liu J, Li J, Yang J, Liu J. Isolation and identification of novel H3N8 subtype avian influenza virus and study on its pathogenicity in chickens[J]. Journal of China Agricultural University, 2022, 27(08):131-139
7. Sit THC, Sun W, Tse ACN, Brackman CJ, Cheng SMS, Tang AWY, Cheung JTL, Peiris M, Poon LLM. Novel Zoonotic Avian Influenza A(H3N8) Virus in Chicken, Hong Kong, China[J]. Emerg Infect Dis, 2022, 28(10):2009-2015
8. Wen F, Zhang X, Guo J, Liang Z, Cheng Q, Wang C, Yu H, Du Y, Huang S, Li J, Li Y. Emergence of H3N8 avian influenza viruses possessing tri-basic hemagglutinin cleavage sites in China[J]. J Infect, 2022, 85(4):e112-e114
9. Gall A, Hoffmann B, Harder T, Grund C, Beer M. Universal primer set for amplification and sequencing of HA0 cleavage sites of all influenza A viruses[J]. J Clin Microbiol, 2008, 46(8):2561-7
10. Gao R, Zheng H, Liu K, Ji Z, Cai M, Gu M, Hu J, Liu X, Hu S, Wang X, Liu X. Genesis evolution and host species distribution of influenza A (H10N3) virus in China[J]. J Infect, 2021, 83(5):607-635
11. Spekreijse D, Bouma A, Koch G, Stegeman JA. Airborne transmission of a highly pathogenic avian influenza virus strain H5N1 between groups of chickens quantified in an experimental setting[J]. Vet Microbiol, 2011, 152(1-2):88-95
12. Luk GS, Leung CY, Sia SF, Choy KT, Zhou J, Ho CC, Cheung PP, Lee EF, Wai CK, Li PC, Ip SM, Poon LL, Lindsley WG, Peiris M, Yen HL. Transmission of H7N9 Influenza Viruses with a Polymorphism at PB2 Residue 627 in Chickens and Ferrets[J]. J Virol, 2015, 89(19):9939-51
13. Zhong L, Wang X, Li Q, Liu D, Chen H, Zhao M, Gu X, He L, Liu X, Gu M, Peng D, Liu X. Molecular mechanism of the airborne transmissibility of

H9N2 avian influenza A viruses in chickens[J]. J Virol, 2014, 88(17):9568-78

14. Gaymard A, Le Briand N, Frobert E, Lina B, Escuret V. Functional balance between neuraminidase and haemagglutinin in influenza viruses[J]. Clin Microbiol Infect, 2016, 22(12):975-983