One Health Advances

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Surveillance and characterization of avian-origin H3N2 canine influenza viruses in 2021 in China

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Abstract

Avian-origin H3N2 canine influenza virus (CIV) is one of the most prevalent influenza virus subtypes in dogs worldwide. Previous studies have shown that during the evolution of H3N2 CIV in dogs, its adaptability in mammals increased gradually, suggesting that dogs can serve as a potential intermediate host for cross-species transmission of the avian influenza virus. In this study, we report results from the surveillance and characterization of H3N2 CIVs isolated from animal hospitals and kennels in 2021 in China. We characterized the CIVs' genetic and antigenic variation, receptor-binding specificity, and virulence in mice. The hemagglutinin (HA) phylogenetic result showed that these H3N2 CIVs belonged to Clade 5.1, a clade formed after 2019. Compared to the 2016–2019 strains in China, the 2021 H3N2 CIVs had similar antigenicity and receptor-binding specificity. The pathogenicity in mice was significantly reduced after infection with two 2021 strains, but the replication capacity was similar, suggesting that a virus-host balance might have been established. This report emphasizes the importance of close surveillance and monitoring of H3N2 CIVs in dogs to prevent the emergence of novel influenza viruses with public health threats.

Keywords Canine influenza virus, Genetic characteristics, Receptor binding property, Virulence in mice

Introduction

Dogs are susceptible to influenza virus infections from avian (H3N2 and H5N1), equine (H3N8), or human (pdmH1N1 and H3N2) [1–3]. H3N8 and H3N2 are the dominating subtypes of canine influenza viruses in dog populations worldwide [3]. H3N8 CIV has predominantly circulated in the United States since 2004 [4], and H3N2 CIV is dominant in South Korea and China [3, 5]. Compared to H3N8 CIV, H3N2 CIV exhibits a broader host range, infecting various mammalian animals, including ferrets, guinea pigs, mice, and cats [6–9]. H3N2 CIV was first isolated in 2006 from the Guangdong Province in China and was found to have a high genetic similarity, across all eight gene segments, to H3N2 avian influenza viruses prevalent in aquatic birds found in South Korea [5]. Since 2006, H3N2 CIV has been prevalent in South Korea [3], China [10], and Thailand [11]. In April 2015,

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the H3N2 CIV, which was circulating in Asia, spread to and established circulation in the United States, resulting in respiratory disease in thousands of dogs [12].

Dogs may play critical roles as mixing vessels and reservoirs contributing to various influenza viruses [13]. Serological and virological data have documented sporadic transmission and subclinical infection in dogs with human influenza viruses [14]. Research also revealed a novel H3N1 virus isolated from infected dogs that resulted from the reassortments of a human-origin H1N1 influenza virus and an avian-origin H3N2 CIV [2]. A novel H3N2 CIV containing the polymerase acidic (PA) segment from the H9N2 avian influenza virus was isolated from a dog in South Korea in 2015 [7].

In addition, canines may serve a role in the adaptation of avian influenza viruses in humans. Our previous studies showed that H3N2 CIV recognized human-like α-2,6-linked sialic acid and acquired a 100% transmission rate via respiratory droplets in a ferret model, suggesting that dogs are potential intermediate hosts for avian influenza viruses to adapt to humans [15]. These studies highlighted the importance of continuous surveillance of H3N2 CIVs in dogs.

For this 2021 study, we focused on animal hospitals and kennels in Beijing and Hainan Province to monitor H3N2 CIV's circulation. We collected throat swabs from 162 dogs with respiratory symptoms and isolated viral samples to characterize genetic variation, antigenicity, receptor-binding specificity, and replication capability in mammals. To better understand the evolution of the 2021 strains, we compared them with strains isolated in previous years.

Results

Isolation of viruses and collection of clinical data

From January to December 2021, we collected throat swab samples from 162 dogs with signs of respiratory disease from animal hospitals and kennels in Beijing and Hainan Province. Seven samples (4.32%) were H3N2 positive, including six strains sampled from Beijing and one from the Hainan province.

Clinical observations indicated that infected dogs displayed symptoms of upper respiratory tract infections and bronchopneumonia, including coughing and rhinorrhea (Table 1). Clinical auscultation revealed rough breathing sounds, and thoracic radiographs showed increased bronchial density. Dogs received treatment with antibiotics chosen based on drug sensitivity tests, along with interferon, expectorants, and antitussives. The majority of dogs recovered within 1–2 weeks.

Phylogenetic analysis and molecular characterization

To understand the evolution of the isolated H3N2 CIVs, the eight gene segments of each isolated CIV were sequenced. The percent sequence identities between each pair of H3N2 CIVs isolated in this study were over 99.0% for each of the eight genes: HA (99.6%–100.0%), neuraminidase (NA) (99.3%–100.0%), polymerase basic proteins 2 (PB2) (99.8%–100.0%), polymerase basic proteins 1 (PB1) (99.8%–100.0%), PA (99.8%–100.0%), nucleoprotein (NP) (99.9%–100.0%), matrix (M) (99.3%–100.0%) and non-structural protein (NS) (99.6%–100.0%).

We further performed a phylogenetic analysis on seven viruses using RAxML, along with 80 representative viruses from GenBank and GISAID based on isolation time and location. The HA gene segment of all seven H3N2 CIVs isolated in this study fell into the Clade 5.1, a clade formed after 2019 in China (Fig. 1 and Fig. S1). The NA and six internal gene segments are all in the same clade as the segments of H3N2 CIVs isolates after 2016.

Then, we investigated the molecular characteristics of the viruses isolated in 2021. We found that compared with strains isolated in China, the Cn/BJ/0501/21 and Cn/BJ/0510/21 strains had mutations at position 23 (E to K) in HA, position 464 (D to N) in PB1, and position 643 (A to T) in PB1. Additionally, all strains isolated in 2021 had mutations at position 598 (A to T) and position 340 (K to R) in the PB2 protein.

Table 1 Avian-origin H3N2 canine influenza cases sampled in China in 2021

Geographic location	Month	Isolate	Canine species	Canine age (months)	Canine sex	Disease history
Beijing	Jan	A/canine/Beijing/0118/2021	Golden retriever	3	Female	Sneezing and rhinorrhea
Beijing	May	A/canine/Beijing/0501/2021	Border collie	2	Male	Coughing and rhinorrhea
Beijing	May	A/canine/Beijing/0510/2021	Toy poodle	108	Male	Sneezing and coughing
Beijing	May	A/canine/Beijing/0511/2021	Japanese spitz	72	Male	Sneezing and coughing
Hainan	May	A/canine/Hainan/0515-W17/2021	Labrador retriever	2	Female	Coughing, vomiting and diarrhea
Beijing	Aug	A/canine/Beijing/JC2538/2021	German shepherd	24	Male	Rhinorrhea
Beijing	Aug	A/canine/Beijing/JC2550/2021	German shepherd	120	Male	Rhinorrhea

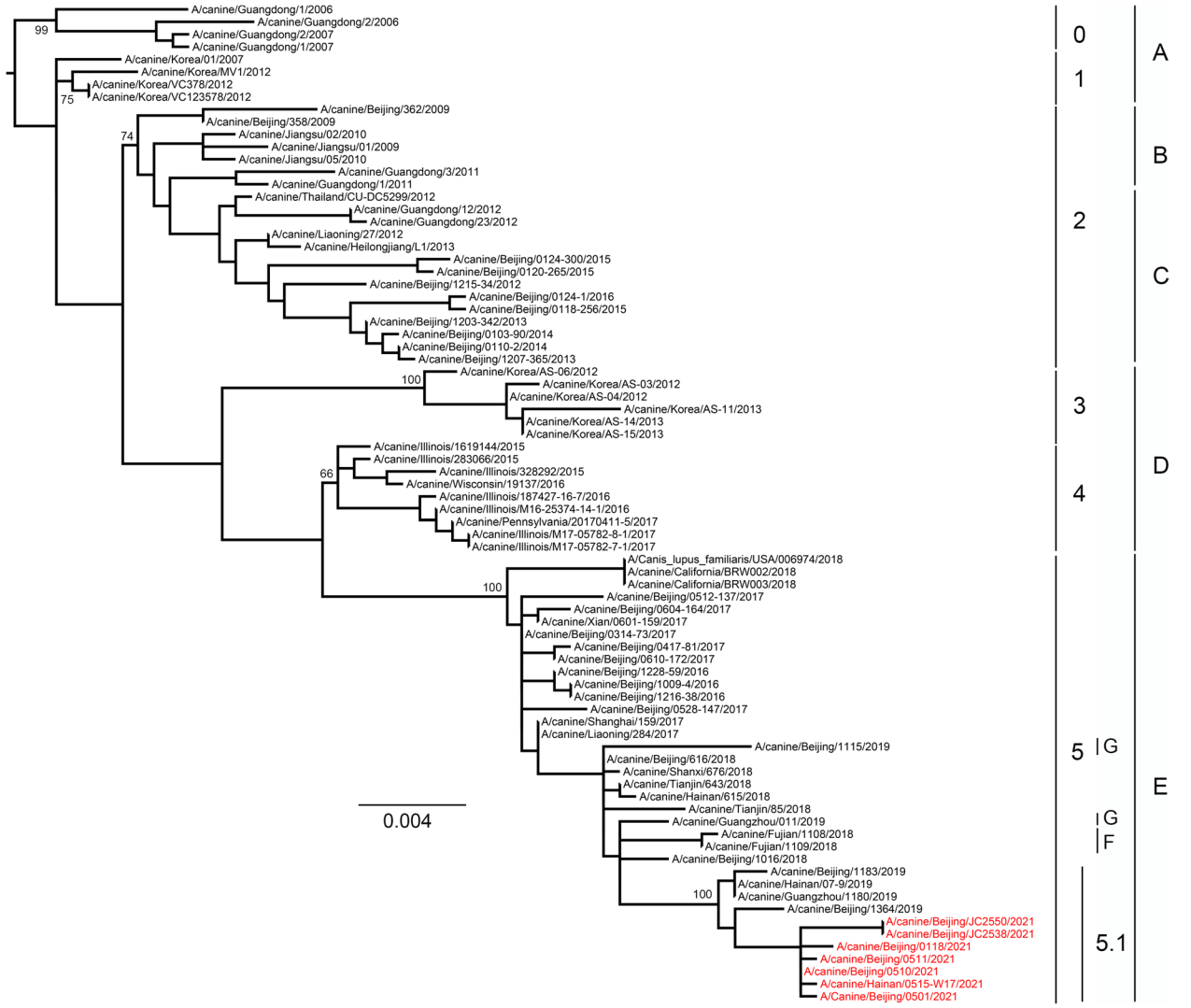

Fig. 1 The phylogenetic tree of the hemagglutinin (HA) segment of H3N2 CIVs. The tree was constructed in maximum likelihood under the GTR + gamma + I model. The 2021 isolates involved in this study are highlighted in red. Names of the viral sequences downloaded from databases were in black, which included 37 strains submitted by our laboratory. A, B, C, D, E, F, and G represent different antigen groups of H3N2 CIVs, respectively. Scale bar is in units of nucleotide substitutions per site

Antigenic analysis

Our previous study showed that seven antigenic groups (A–G) were formed for the global H3N2 CIVs. To determine the antigenic variation of the H3N2 CIVs isolated in 2021, we analyzed their antigenicity using a panel of polyclonal sera raised against H3N2 viruses with previously identified antigenic groups. As shown in Table 2, the H3N2 CIVs isolated in 2021 reacted best with antiserum against viruses of the antigenic group E over other antigenic groups. The antigenic group E mainly consists of viruses isolated in 2016–2019 in China. They all belong to Clade 5 and 5.1 and are currently the dominant prevalent strains in China.

Receptor binding specificity of H3N2 CIVs

Avian viruses exhibit a preference for binding to sialosides linked to the terminal oligosaccharide by an α-2,3 bond (referred to as the avian receptor), while human strains favor the α-2,6-linked sialosides receptor (referred to as the human receptor) [16]. Our previous study showed that the H3N2 CIVs isolated in 2016–2019 in China could bind to both the α-2,6-linked glycans and α-2,3-linked glycans [15]. In this study, we found that the H3N2 CIVs isolated from 2021, represented by Cn/BJ/0118/21, Cn/BJ/0501/21, and Cn/BJ/0510/21, also showed dual binding specificity to both α-2,3- and α-2,6-linked sialosides (Fig. 2).

Table 2 Antigenic analysis of H3N2 CIVs sampled in 2021

Antigenic group and virus	Human BJ/1230/16	A Cn/GD/1/06	B Cn/BJ/362/09	C Cn/BJ/265/15	D Cn/IL/M17-05782-7-1/17	E Cn/BJ/137/17	F Cn/FJ/1109/18	G Cn/GZ/011/19
BJ/1230/16(Human)[a]	**1280†**	40	40	160	20	20	80	160
A								
Cn/GD/1/06 [a]	<	**1280†**	80	40	<	160	40	80
B								
Cn/BJ/362/09 [a]	<	320	**1280†**	160	<	160	160	160
C								
Cn/BJ/265/15 [a]	<	640	160	**1280†**	<	160	80	160
D								
Cn/IL/M17-05782-7-1/17 [a]	<	20	<	<	**1280†**	<	<	<
E								
Cn/BJ/137/17 [a]	<	320	40	320	<	**1280†**	80	80
Cn/BJ/1183/19 [a]	<	320	40	320	<	1280	320	320
Cn/BJ/0118/21 [b]	<	160	40	160	<	1280	80	160
Cn/BJ/0501/21 [b]	<	320	80	320	<	640	160	80
Cn/BJ/0510/21 [b]	<	160	40	320	<	1280	80	160
F								
Cn/FJ/1109/18 [a]	<	160	40	80	<	80	**1280†**	160
G								
Cn/GZ/011/19 [a]	<	320	40	320	<	160	160	**1280†**

Hemagglutinin Inhibition (HI) titers (are the inverse of the highest dilution that inhibited hemagglutination. Cells in black: high titers (≥ 640). Cells in gray: moderate HI titers (160, 320). Low HI titers (40, 80) are not shaded. " < ": Titers < 10. †Homologous titer. [a] Viruses identified previously were selected as reference strains for different HI groups. [b] Isolated strain

Reduced pathogenicity in mice of some H3N2 CIVs in 2021

To systematically evaluate the replication and pathogenicity of the H3N2 CIVs in mice, we selected 16 H3N2 CIVs isolated from China, including two viruses from each year in 2009, 2015, 2016, 2017, 2018, 2019, one from 2013, and three from 2021. Mice were inoculated with 10^6 EID_{50} (50% egg infectious dose) of these viruses and showed diverse maximum body weight loss during the observation period (Fig. 3). Five viruses from 2009, 2013, and 2015 in Clade 2 caused no significant weight loss, but some mice did gain body weight during infection. Six viruses from 2016–2018 in Clade 5 caused 8.1%–17.2% maximum body weight loss in mice, two viruses from 2019 in Clade 5.1 caused 6.5%–7.1% maximum body weight loss, and one virus (Cn/BJ/0118/21) from 2021 in Clade 5.1 caused 5.0% maximum body weight loss in mice, but the other two viruses (Cn/BJ/0501/21 and Cn/BJ/0510/21) from 2021 in Clade 5.1 caused no significant weight loss in mice during infection.

All 16 viruses could replicate in the lungs of mice, with titers ranging from 3.5 to 6.5 $\log_{10}EID_{50}$. The viruses isolated from 2009, 2013, and 2015 in Clade 2 had more than one log lower titers than those isolated from 2016–2021 in Clade 5 and 5.1. Additionally, a histopathological examination of the lungs from virus-infected mice was performed at 4 dpi (Fig. 4 A–J). The lungs infected with Cn/BJ/362/09 (Clade 2) and Cn/BJ/0124–300/15 (Clade 2) had no significant histopathological changes. Lungs infected with Cn/GZ/1180/19(Clade 5.1) and Cn/BJ/0118/21 (Clade 5.1) showed moderate histopathological changes and lungs infected with Cn/BJ/1228–59/16 (Clade 5) and Cn/SH/159/17 (Clade 5) showed severe pathological lesions. Importantly, the lungs infected with Cn/BJ/0501/21 (Clade 5.1) and Cn/BJ/0510/21 (Clade 5.1) also had no significant histopathological changes. These results highlighted that these two H3N2 CIVs isolated in 2021 may have established a virus-host balance and evolved with better replication fitness with weakened symptoms.

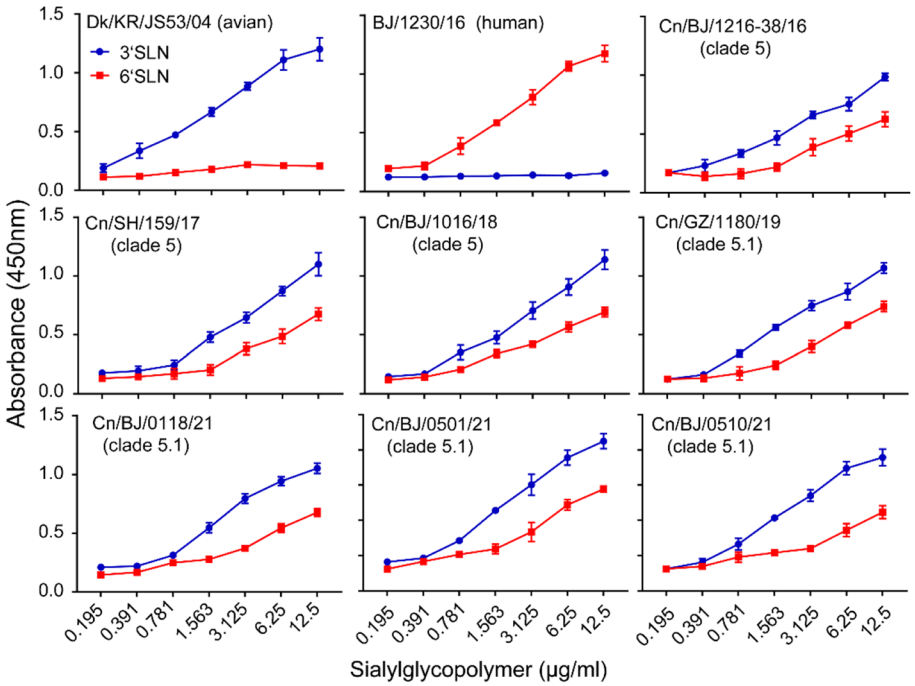

Fig. 2 Characterization of receptor-binding properties of H3N2 CIVs. Direct binding of the virus to sialylglycopolymers containing either 2,3-linked (blue) or 2,6-linked (red) sialic acids was tested. Data showed here are the means from three replicates; error bars show the standard deviations

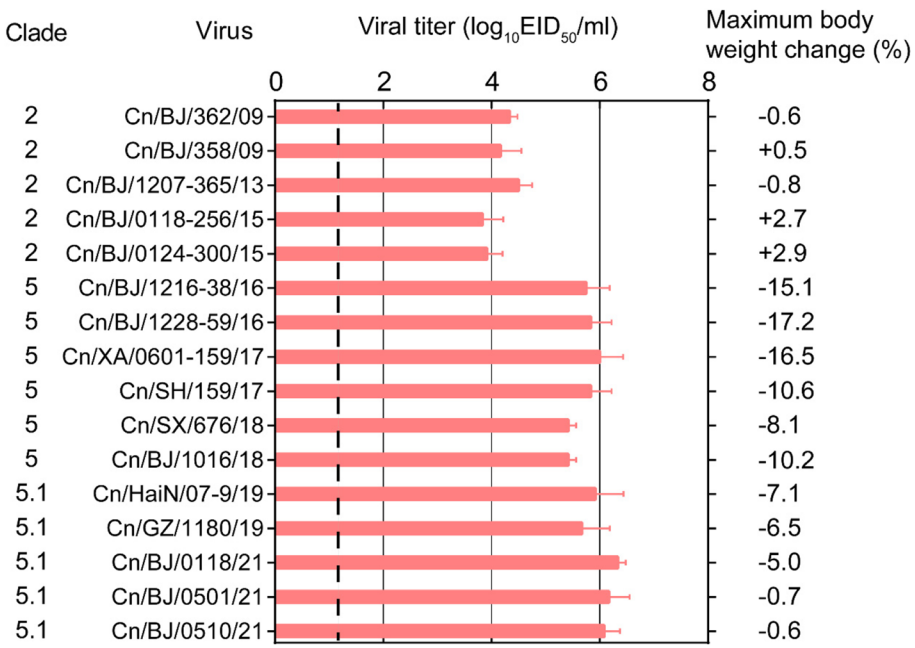

Fig. 3 Replication and virulence of H3N2 CIVs in mice. Virus titers in lungs of mice on day 4 p.i. with 10^6 EID_{50} of test virus. Data showed are the mean titers from three mice; error bars show the standard deviations. The dashed line defines the lower limit of detection

Discussion

H3N2 CIV is considered to have originated from avian influenza viruses through adaptive evolution [17]. In the previous study we found that during its adaptation to dogs, H3N2 CIV posed an increased threat to human health. These viruses gained the ability to

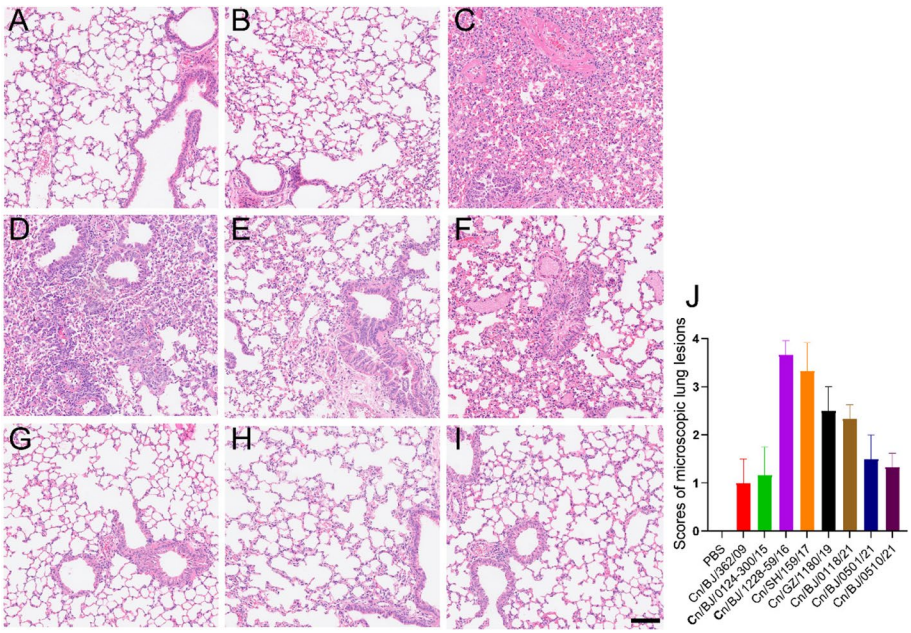

Fig. 4 Histological lesions caused by H3N2 CIVs in the lungs of mice. Mice were euthanized on day 4 p.i. with 10^6 EID_{50} of test virus, and the lungs were collected for pathological study. Lungs of (**A**) Cn/BJ/362/09, **B** Cn/BJ/0124-300/15, **G** Cn/BJ/0501/21, and (**H**) Cn/BJ/0510/21 showed no significant histopathological changes (H&E staining). **E** Cn/GZ/1180/19 and **F** Cn/BJ/0118/21 virus-inoculated animal showed moderate histopathological changes (H&E staining), whereas the lungs of (**C**) Cn/BJ/1228-59/16 and **D** Cn/SH/159/17 virus-inoculated mice showed severe pathological lesions (H&E staining). **I** The lung samples of mice inoculated with PBS. Scale bar, 200 μm. **J** Microscopic lesions were counted

recognize the human-like SAα2,6-Gal receptor, demonstrated a progressively increased ability to replicate in human airway epithelial cells, and achieved a 100% transmission rate in a ferret model [15]. Therefore, we suggested that dogs are potential intermediate hosts by which some avian influenza viruses have adapted to humans.

In the present study, we monitored H3N2 CIVs in dogs. Seven throat swab samples from 162 dogs with signs of respiratory disease were H3N2 CIV positive. The isolated H3N2 CIVs showed similar genetic and antigenic characteristics, receptor-binding specificity, and replication capability in mice compared to the viruses from 2016 to 2019. However, two strains isolated from 2021 exhibited weakened pathogenicity in mice compared to the 2016–2019 viruses, indicating the establishment of a virus-host balance.

Our previous study showed an increasing positive rate of H3N2 CIV in the dog population in China, from 1.98% in 2012 to 10.85% in 2019, and a sharp increase since 2016. However, since the outbreak of COVID-19 in late 2019, the influenza virus positive rate in dogs has significantly decreased (4.32% in 2021). In January 2020, China took rapid actions to prevent and control COVID-19 by implementing emergency health policies including restrictions on public activities and the closure of public places [18]. As a result, the interactions among dogs were also reduced. Additionally, according to a white paper on the nation's pet industry in 2021 released by petdata.cn, the number of cats has outnumbered dogs, resulting in a decrease in the number of pet dogs. Moreover, under the control and governance of the Chinese government, the number of stray dogs has also shown a noticeable downward trend.

Our previous study found that in both dogs and ferrets, the replication and transmission of H3N2 CIVs isolated from 2016 to 2019 gradually increased [15]. In this study, we systematically compared the replication capacity and pathogenicity of the CIVs over the past decade in mice. We found that recently isolated CIVs (year 2021) exhibited enhanced replication capacity in mice, a result consistent with our prior observations in dogs and ferrets. However, compared to strains isolated from 2016 to 2019 in China, the pathogenicity of some strains isolated in 2021 was significantly reduced while the replication capacity was similar. The PB2-A643T, HA-E23K, and HA-D464N mutations were observed in the attenuated 2021 strains, suggesting that further validation is necessary to test if these mutations relate to reduced pathogenicity.

Our results indicate that H3N2 CIVs may have established a virus-host balance. In response to influenza virus infection, the host immune system is often activated to prevent viral replication [19, 20]. Meanwhile,

emerging viruses would undergo host-specific selection after transferring to new hosts [21]. Viruses with a better replication capability are prone to be selected and become dominant after competing with many other candidate strains. However, a better replication capability is often accompanied by increased pathogenicity [22]. After pathogenicity reaches a certain level, it needs to become attuned to establish a virus-host balance [23–25]. During the early phase of the 2009 influenza pandemic, the global genetic diversity of influenza viruses increased rapidly as a result of transmissions in the predominantly naïve human population [26]. In subsequent waves of the 2009 pandemic, host adaption led to increased viral fitness and transmission. Consequently, the reduced pathogenicity of A/H1N1pdm09 viruses emerged [27] and eventually reached fixation in the global virus population [28]. Likewise, following the appearance of SARS-CoV-2, five distinct variants of concern have been recognized: Alpha, Beta, Gamma, Delta, and Omicron. [29]. The Omicron variant emerged in November 2021 and rapidly transmitted in the human population [30]. Attenuated pathogenicity of the Omicron variant, compared to the original strain of SARS-CoV-2, was identified in several animal models [29, 30].

In summary, our results from this study suggested that the current circulating H3N2 CIVs are evolving to gain a virus-host balance with better replication capability and causing mild symptoms, posing increased public health threats. Our study heightens the importance of close surveillance and monitoring of H3N2 CIVs in dogs to prevent the emergence of novel influenza viruses with the potential to cause public health crises.

Materials and methods

Ethics statements

This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The animal study protocols were approved by the Committee on the Ethics of Laboratory Animals of China Agricultural University.

Virus isolation and identification

During January 2021 to December 2021, we collected 162 throat swabs from dogs with signs of respiratory disease in animal hospitals and kennels in Beijing and the Hainan province to monitor for CIV H3N2 epidemics and virus evolution. For virus isolation, throat swabs were taken and placed in 1.0 mL transmission medium (50%(vol/vol) glycerol in PBS) containing antibiotics, as previously described [31]. We performed the amplification of the M gene through real-time reverse transcription PCR. Subsequently, virus isolates were isolated and identified following previously established methods [10]. Each sample that tested positive through PCR was individually inoculated into 10-day-old embryonated chicken eggs, allowing incubation for 72 h at 35 °C. Subsequently, the allantoic fluid was collected and tested for hemagglutination activity using 1% chicken red blood cells (cRBCs). If the hemagglutination activity of a sample was negative, all eight gene segments were deep sequenced. If the hemagglutination activity of the sample was positive, all eight viral segments were determined by direct sequencing of the PCR products. The primer sequences and amplification conditions can be provided by the authors upon request. The viruses were stored in a −80 °C freezer.

Phylogenetic and molecular analyses

Seven H3N2 CIV isolates were used for genetic and phylogenetic analyses in this study. A total of 80 representative viruses were collected from the NCBI and GISAID databases, considering factors such as isolation time and location. Phylogenetic analyses were performed on the alignment regions containing the least gaps across sequences. RAxML was used to construct the maximum likelihood phylogenies for each segment [32], via CIPRES Science Gateway; 1,000 bootstrap replicates were run, and GTR + gamma + I was used as the nucleotide substitution model.

Antigenic analyses

The antigenic characterization of H3N2 viruses, isolated in this and previous studies, was performed using hemagglutination inhibition (HI) assays. Antisera from ferret infected with H3N2 viruses were used to characterize the viruses; the following viruses were included: BJ/1230/16, Cn/GD/1/06 (group A), Cn/KR/01/07 (group A), Cn/BJ/358/09 (group B), Cn/BJ/362/09 (group B), Cn/BJ/256/15 (group C), Cn/BJ/265/15 (group C), Cn/KR/AS-03/12 (group D), Cn/IL/M17-05782–7-1/17(group D), Cn/BJ/137/17 (group E), Cn/BJ/147/17 (group E), Cn/FJ/1109/18 (group F), Cn/GZ/011/19 (group G), and Cn/BJ/1115/19 (group G). The HI test was performed as previously described [33].

Receptor-binding specificity assay

A-2,6 glycans (6′SLN: Neu5Acα2-6Galβ1-4GlcNAcβ-SpNH-LC-LC-biotin) and α-2,3 glycans (3′SLN: Neu5Acα2-3Galβ1-4GlcNAcβ-SpNH-LC-LC-biotin) were kindly provided by the Consortium for Functional Glycomics (Scripps Research Institute, Department of Molecular Biology, La Jolla, CA, USA). Receptor-binding specificity was analyzed with a solid-phase binding

assay as previously described [34]. Briefly, plates were coated with serial dilutions of 3′SLN and 6′SLN overnight at 4 °C. Then, the glycan solution was removed, and the plates were blocked at room temperature for 1 h, and incubated with a solution containing 2^6 HA units of each influenza virus type at 4 °C for 10 – 12 h. After washing, the plates were incubated with chicken serum against influenza viruses. The plates were then incubated with HRP-linked goat anti-chicken antibody (Sigma-Aldrich) for 2 h at 4 °C. Finally, the plates were incubated 3,3',5,5'-Tetramethylbenzidine (TMB) two-component substrate solution (Soliabio, Beijing, China) for 10 min at room temperature. The reaction was stopped with 1 M H_2SO_4 and the absorbance was read at 450 nm.

Mouse experiments

Groups of 6 to 8-week-old female BALB/c mice (Boehringer Ingelheim, Beijing, China) were anesthetized with 20 mg/g tiletamine-zolazepam (Zoletil; Virbac SA, Carros, France) and inoculated intranasally with 50μL of 10^6 EID_{50} of each virus diluted in PBS. Control mice were inoculated with 50μL of PBS. Three mice from each group were euthanized at 4 dpi for virus titration and histology. Lung microscopic lesions were blindly evaluated on a scale from 0 to 4 in five random fields to assess the distribution and severity of interstitial pneumonia. The remaining three mice were monitored daily for 14 days for weight loss and mortality.

Accession numbers

The viral nucleotide sequences are available from the GenBank using the accession numbers OR473194-OR473249.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44280-023-00034-8.

Additional file 1: Figure S1. Phylogenetic relationships of fully sequenced H3N2 CIVs sampled in China in 2021.

Acknowledgements

We thank all authors and laboratories to assist this project.

Authors' contributions

M.Y., R.W., Y.P., T.Z., and Y.L. collected the data. M.Y., Y.S., J.M., S.V., Z.W. and M.Z. analyzed and interpreted the data. M.Y., Z.W. and M.Z. wrote the manuscript. All authors reviewed, revised, and approved the final manuscript.

Funding

This work was supported by the Higher Education Discipline Innovation Project.

Availability of data and materials

All data and materials are available for free for non-profit institutions.

Declarations

Ethics approval and consent to participate

Ethical approval was reviewed and granted by China Agricultural University Animal Ethics Committee document (No. AW01017102-2).

Consent for publication

All authors reviewed, revised, and approved the final manuscript.

Competing interests

No potential conflict of interest was reported by the authors.

Received: 10 September 2023 Revised: 25 November 2023 Accepted: 28 November 2023
Published online: 11 January 2024

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