Genetic evolution of class I Newcastle disease virus from poultry at live bird markets in Eastern China

Xiaolong Lu

Yangzhou University <https://orcid.org/0000-0001-9849-1685>

Xiaoquan Wang

Yangzhou University

Yifan Sun

Yangzhou University

Xin Wang

Yangzhou University

Lin Feng

Yangzhou University

Yu Chen

Yangzhou University

Tiansong Zhan

Yangzhou University

Naiqing Xu

Yangzhou University

Tianxing Liao

Yangzhou University

Jiao Hu

Yangzhou University

Zenglei Hu

Yangzhou University

Min Gu

Yangzhou University

Shunlin Hu

Yangzhou University

Xiaowen Liu

Yangzhou University

Xiufan Liu (✉ xfliu@yzu.edu.cn)

Keywords: Newcastle disease virus, Epidemiological investigation, Live bird markets, Commercial farms, Terrestrial birds, Class I, Sub-genotype 3c

Posted Date: July 16th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-41422/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Live bird markets (LBMs) serve as a natural reservoir for class I Newcastle disease virus (NDV) and play an important role in viral evolution and spread. LBMs and commercial farms are the main sources of daily poultry products. However, limited studies are available for the class I NDV circulating at LBMs and commercial farms. In this study, significant progress has been made in sample detection and genetic analysis at LBMs and commercial farms in Eastern China.

Methods: A long-term epidemiological investigation at LBMs and commercial farms in Eastern China was conducted. We sequentially conducted the collection of samples, virus isolation, RNA extraction, RT-PCR, and phylogenetic analysis. We also analyzed class I NDVs deposited in NCBI during 2002–2018 from China in terms of the host, genotype, time, and functional domains.

Results: Here we report that class I NDVs continue to circulate in LBMs. Class I NDVs were detected at a high prevalence in chickens (23/26) but were seldom detected in waterfowl (3/26) at LBMs. In contrast, class I NDVs were rarely detected in commercial chickens but were present in commercial waterfowl at a certain frequency before they were brought to the LBMs. This observation suggests that the mixture of poultry and the conventional housing at LBMs cause the spread of class I NDVs. Sequence analysis of Class I NDV genomes deposited in NCBI during 2002–2018 from China revealed a high prevalence of class I NDVs in terrestrial birds (82.7%), which was much higher than in water birds (17.3%). In addition, we found that the class I NDVs predominantly belonged to the sub-genotype 3c and rarely evolved into a new sub-genotype such as the 3d. Furthermore, the epidemic sub-genotype 3c seems to be under ongoing evolution.

Conclusions: Our study provides evidence that the traditional feeding mode at LBMs causes the spread of class I NDVs and poses a great risk of NDV epidemics in the poultry industry in Eastern China. The sub-genotype 3c is circulating at LBMs and currently the terrestrial bird is the predominant host of class I NDVs.

Background

Newcastle disease virus (NDV), also termed as avian paramyxovirus type 1 (APMV-1), belongs to the genus Orthoavulavirus in the family Paramyxoviridae according to the International Committee on Taxonomy of Viruses (ICTV) [1]. NDV is a single-stranded, negative-sense, non-segmented RNA virus of approximately 15.2 kb. From the NDV genome (3' to 5' terminus), six proteins are produced: nucleocapsid protein (NP gene), phosphoprotein (P gene), matrix protein (M gene), fusion protein (F gene), hemagglutinin-neuraminidase protein (HN gene), and the RNA-dependent RNA polymerase protein (L gene) [2]. Based on the genome size and phylogenetic analysis of the F gene, NDV can be divided into two groups: class I and class II [3]. Class I is exclusively avirulent and can be subdivided into nine genotypes (1–9). In comparison, class II is subdivided into eighteen genotypes (I–XVIII) based on the

partial sequence of the F gene (47–420 nt). In addition to the classical classification methods, two modified classification methods have been introduced, first in 2012 and then in 2019 [4, 5].

The class I NDV, which with 15,198 nucleotides has the longest genome of APMV-1[3], is present in domestic and wild waterfowl widely [2, 6]. Compared with the full genome sequences of Class II NDV early lineage strains (15,186 bp), class I NDV has a 12-bp insertion in the phosphoprotein gene [7]. Waterfowl, the natural reservoir of NDVs, has the potential to spread the virus in the environment and within their susceptible hosts. It results in frequent ND outbreaks and subsequent economic losses [8–10]. Class I NDVs were originally recovered from both domestic and wild birds sampled in North America and Eurasia since at least the 1970s [6, 11, 12]. Then, it was reported that class I NDVs are also widely common in the USA and China [11, 13, 14]. The susceptible hosts of class I NDVs are quite manifold such as domestic poultry (chickens, ducks, geese, and pigeons) and wild birds (black swan, peafowl, egret, and heron) [6, 11, 13, 15]. Also, the susceptible hosts in different genotypes are slightly different [3]. Class I viruses are generally lentogenic in chickens while there is a known mutant and historically it has been recovered from waterfowl (family: Anatidae) and shorebirds [16]. Furthermore, it is noteworthy mentioning that a virulent class I NDV causes the 1990 Ireland ND outbreak [11], and lentogenic NDVs, including class I, can enhance virulence through chicken or chicken embryo passages [17]. Most of the avirulent NDVs isolated from the LBMs belong to the class I clade [2, 15, 18, 19]. Therefore, an in-depth epidemiological investigation of class I NDVs is significant to understand the spread and variation of the class I NDVs and predict future trends. However, little is known about the origin and the genetic evolution of class I NDVs at LBMs. Despite the epidemiology of NDV in several regions of China being well characterized [20–22], information for infection in Eastern China has rarely been reported in recent years.

In this study, we investigated the prevalence of class I NDVs at LBMs and commercial farms in Eastern China. LBM and farm poultry data were combined to analyze and their role in the transmission of class I NDVs was highlighted. Besides, we systematically investigated the genetic evolution of class I NDVs deposited in NCBI during 2002–2018 from China. Taken together, this study provides theoretical guidelines to evaluate the prevalence of class I NDVs in China.

Materials And Methods

Collection of samples

We administered a long-term survey of settled LBMs and commercial farms in Eastern China during 2017–2018. The avian samples mainly came from several provinces in Eastern China, such as Jiangsu, Anhui, Shandong, Zhejiang, and samples from other areas were sporadic. Monthly, we collected pooled oropharyngeal and cloacal swabs from randomly- selected poultry at each LBM. We also collected a few environmental samples at LBMs. Similarly, we collected samples from commercial poultry regularly. Then samples were transported at 4°C, immediately aliquoted, and stored at –80°C until testing.

In addition to collecting the above samples, our laboratory conducted a long-term epidemiological investigation in Eastern China during 2002–2016 and isolated several class I NDVs. For the

comprehensiveness of data, the previous data were combined and analyzed together.

Virus isolation, RNA extraction, RT-PCR and sequencing of the F gene

The viruses isolated from LBMs and commercial farms were identified, and the virus isolation method was previously described [19]. The swab samples were frozen, thawed three times on dry-ice, and squeezed. Then the supernatants were collected after centrifugation (5 min, 8,000 rpm) at 4°C. The supernatant was inoculated with 10-day-old specific-pathogen-free (SPF) chicken embryos (Beijing Merial Wetong Experimental Animal Co., Ltd.), and the allantoic fluid was collected from incubated eggs at the appointed time as described previously [19]. Next, viruses were identified by hemagglutination (HA) and hemagglutination inhibition (HI) experiments (chicken NDV standard positive serum prepared by our lab). The virus stock was stored at -80°C until use. Sample collection and all experiments in the present study were performed in negative-pressure isolators with HEPA filters in a biosafety level 3 (BSL3) animal facility by the institutional biosafety manual.

Viral RNA extraction and RT-PCR were performed as described previously [23]. PCR was performed using NDV F gene-specific consensus primers: forward primer (4494–4517): 5'-CGTAGAAAAACACGGGTAGAAGA-3' and reverse primer (5429–5452): 5'-CAGGTAGGTRGCACGCATATTATT -3'. The F genes of all the isolates were sequenced from the PCR products by Sanger sequencing (Genscript, Nanjing) and submitted to GenBank (GenBank IDs: listed in additional file 1).

Phylogenetic evolution analysis

Nucleotide sequence editing, analysis, and alignment were performed using the Clustal W multiple alignment method in the BioEdit (BioEdit Sequence Alignment Editor, version 7.2.5) and MEGA 7 software (Molecular Evolutionary Genetics Analysis, Version 7.0.21). We used the classical classification method because the collected sequences were concentrated in 47–420 nt. Phylogenetic trees were constructed using the neighbor-joining method in MEGA 7 based on the F partial gene (374 bp). To estimate evolutionary rates and distances of different sub-genotypes of genotype 3 in F genes, the molecular analysis was performed based on the pairwise distances implemented in MEGA 7 (nucleotide: maximum composite likelihood).

There are 241 strains deposited in NCBI, and the information about these nucleotide sequences is given in additional file 1. The F gene sequences of NDVs (n = 241) from the NCBI database (<https://www.ncbi.nlm.nih.gov/>) were added to the phylogenetic analysis.

Analyses of class I NDVs in terms of the host, genotype and time

We elucidated the relationship among the host, genotype, and time of class I NDVs. Class I NDVs isolated in China during 2002–2018 were compared according to the host, genotype, and time. Stacked bar charts were drawn by the GraphPad Prism 7.00 software. We also studied the distribution of the host and time in different genotypes presented as phylogenetic trees. The evolutionary history was inferred by the neighbor-joining method and evolutionary analyses were conducted in MEGA 7. Then, phylogenetic trees were edited and decorated by the Dendroscope (3.5.7) software.

Analyses of key functional domains in F protein of epidemic genotype

To further investigate the current epidemic genotype, we analyzed the amino acids in key functional domains of sub-genotype 3c NDVs. The functional areas included the signal peptide, fusion protein, transmembrane domain, and N-linked glycosylation sites. Protein sequences were predicted and analyzed in MEGA 7.

Results

Prevalence of class I NDVs in chickens at LBMs but not in commercial chickens

We collected 1,618 samples from poultry at LBMs during 2017–2018, including chicken swab samples, duck swab samples, goose swab samples, pigeon swab samples, and environmental samples. Twenty-six class I-positive samples were isolated from LBMs, and the other three strains were highly homologous to the vaccine strain La Sota. We detected class I NDVs in 23 (1.42%) isolates of chicken and 3 (0.19%) isolates of goose. The results revealed that the positive samples mainly belong to the class I branch at LBMs. We also collected samples from commercial farms, including commercial chickens and ducks. There was a certain separation rate from commercial ducks, and Muscovy ducks were more susceptible to NDV, while there was no NDV isolated from commercial chickens (Table 1). In addition to the recent isolates, our lab isolated 308 class I strains at LBMs in Eastern China during 2002–2016, and 106 strains were further studied. Therein, the 106 strains came from chickens (52/106), ducks (45/106), and geese (9/106) (Table 2). Besides, the isolation rate in the colder months would be higher than in the hotter months, which was similar to the influenza virus isolation changes because of seasonal changes (relevant information was not displayed).

To avoid repetitive sequences, we screened a fraction of the sequences ($n = 87$) isolated by our lab for the evolutionary genetic analysis. The phylogenetic tree showed that class I isolates were further divided into the genotype 2, the sub-genotypes 3b and 3c (Figure 4), and the cleavage site sequence of F genes was $^{112}\text{E}/\text{G}-\text{R}-\text{Q}-\text{E}/\text{G}-\text{R}-\text{L}^{117}$ (unlisted).

The terrestrial birds and sub-genotype 3c have become the popular host and genotype, respectively, and a new sub-genotype was confirmed

In pursuit of data reliability, we conducted a synthetic analysis of class I NDVs during 2002–2018 in China. The class I NDVs isolated in China were distributed throughout the country, but the class I NDVs focused on the eastern coastal areas (Figure 1). There was a pattern on the host and genetic distribution. For hosts, water birds are thought to provide a natural reservoir for lentogenic NDVs, but the result showed that class I NDVs gradually transitioned from water birds to terrestrial birds (Figure 2 and 5A). For genotypes, the result showed that the isolation rate of the genotype 3 increased with time, and the sub-genotype 3c became the most popular genotype in recent years (Figure 3 and 5B).

According to the phylogenetic tree, the class I NDVs isolated from our laboratory could be divided into the genotype 2 ($n = 13$), 3b ($n = 34$), and 3c ($n = 40$). The homology rates among three genotype groups were 87.3%–92.3% (2 vs 3b), 85.8%–93.2% (2 vs 3c), and 84.3%–94.1% (3b vs 3c). Further analysis showed that the homology rate of the genotype 2 isolates ranged from 94.1% to 100%, and their homology rate was 93.4%–99.2%. The genotype 2 isolates were close to the genetic relationship of the isolates from 2007 to 2011. The homology rate of the sub-genotype 3b isolates ranged from 94% to 100%. Compared with the isolates from 2005 to 2010, their genetic relationship was relatively close, and the homology rate was 93.7%–99.7%. The homology rate of the sub-genotype 3c was 91.3%–100%, and their homology rate was 90.7%–100%. The sub-genotype 3c isolates were close to the genetic relationship of the isolates in 2009–2018,

The phylogenetic tree based on the partial F genes of class I NDVs in China is shown in Figure 4. At present, the genotype 3 could be divided into 3a, 3b, and 3c, while the genetic evolution analysis of the F gene revealed a new branch in genotype 3. The two strains (HQ398796, HQ398797) published in NCBI formed a separate branch. The mean evolutionary distances between the new branch and three established branches, i.e., 3a, 3b, and 3c were 0.044, 0.071, and 0.084, respectively (Table 3). This conformed to the classification criteria for sub-genotypes. Therefore, the new sub-genotype was provisionally named as 3d. Moreover, the sub-genotype 3d might evolve from 3a because of the higher homology with the genotype 3a than 3b and 3c.

Epidemic genotypes or sub-genotypes constantly change through host and time alternation

Genotypes or sub-genotypes were associated with host and time. According to the analysis of the phylogenetic tree, the prevalent genotypes of class I NDV in China were the genotype 2 and 3 during 2002–2018. From the host perspective, the waterfowl was the susceptible host of the genotype 2. In comparison, the susceptible hosts of the genotype 3 were much more abundant. Each sub-genotype of the genotype 3 was widely found in terrestrial birds, and the sub-genotype carried by water birds was concentrated in the sub-genotype 3b. Besides, the sub-genotype from wild birds was concentrated in the sub-genotype 3c (Figure 5A). From the time perspective, the epidemic sub-genotypes were the sub-

genotype 3a and 3b during the early stages of surveillance (during 2002–2005). At the middle stage of surveillance (during 2006–2009), the popular genotypes were the genotype 2 and sub-genotype 3b. At the end of surveillance (during 2010–2018), the epidemic sub-genotype turned into the sub-genotype 3c (Figure 3 and 5B).

Concerning sub-genotype 3c, terrestrial birds increasingly became the susceptible host, and exogenous viruses disrupted the sequences

According to the phylogenetic tree, the class I NDVs isolated from 2011 to 2018 all belong to the sub-genotype 3c. Although the early isolation rates of water birds and terrestrial birds each accounted for a certain percentage, the terrestrial birds gradually replaced water birds. Besides, the result showed that the homology among the isolates from 2009 to 2018 was higher than with isolates from other years, and there was also a high homology in the isolates with distant years (Figure 4). According to the epidemiological surveillance of the settled LBMs in recent years, most of the NDV isolates were classified into a unified branch. However, some isolates were also merged into a branch with exogenous NDVs, and there was a higher homology among these strains. For example, six isolates in 2018 belong to the same branch with the exogenous strains in 2013. This phenomenon had been observed since 2009 (Figure 4). These results show that exogenous NDVs were constantly invading the LBMs, and class I NDVs were evolving at LBMs.

The tendency for amino acids variation in the signal peptide of the sub-genotype 3c NDVs

We analyzed the evolution of functional regions in the F protein of the sub-genotype 3c NDVs, including the signal peptide, fusion region, transmembrane domain, and N-linked glycosylation sites. The tendency for amino acid variations in these functional domains existed only in the signal peptide of the F protein (Table 4). In detail, there was a variation of amino acids at the 8th, 14th, and 18th sites in the signal peptide: S→G, P→L, M→V.

Discussion

Newcastle disease went through four pandemics, and there have been variant genotypes documented as the epidemic genotype in China and some other Asian countries since the 1990s [24]. Lentogenic NDVs gradually spread through poultry at LBMs, and most of the lentogenic NDVs isolated from LBMs in America and Korea belong to class I clade [25, 26]. In recent years, the isolation rate of class I NDVs at LBMs has significantly risen, and the sub-genotype 3c has gradually become an epidemic strain [22, 27]. Although class I NDVs are genetically conserved and avirulent, these viruses are likely to cause variation and epidemic of ND without any control measures [11].

Class I NDVs, frequently isolated from LBMs [6], were originally found in waterfowls such as ducks and geese, which were the natural reservoirs of avirulent NDVs [15, 20]. In general, class I NDVs were more susceptible to wild birds and water birds [28]. The existence of genetically related class I NDVs in domestic birds and wild birds implied that there were epidemiological connections among these populations [29]. Interestingly, this study showed that class I NDVs were isolated principally from chickens rather than water birds at LBMs. According to the epidemiological survey at LBMs from 2002 to 2018, terrestrial birds gradually became the popular host of class I NDVs after 2014. This result confirmed that the susceptible host of class I NDVs had transitioned from water birds to terrestrial birds at LBMs as a previous study illuminated [11]. We speculated that the emergence of this phenomenon might be related to the polyculture of different birds at LBMs, which may have promoted the circulation of class I NDVs. In response to this problem, we should strengthen the monitoring of the isolated feeding of different poultry flocks at LBMs.

However, we were surprised to find no class I NDV isolated from commercial chickens while there still was a certain virus isolation rate in commercial ducks. This phenomenon interestingly showed that commercial chicken farms were not the cradle of class I NDVs. The monitoring results of LBMs and commercial farms showed that the mixed breeding model at LBMs facilitated the spread of class I NDVs, and water birds carried a steady stream of class I NDVs to infect newly introduced chickens. Many Southeast Asian farmers have been upgrading from the backyard to confined all-in-all-out systems [30, 31]. But some traditional LBMs did not popularize the "all in and all out" model in Eastern China, which might provide an opportunity for virus spread. Therefore, a scientific raising model at LBMs would help control the epidemic of ND.

To obtain a comprehensive analysis of class I NDVs, we also selected all class I NDVs published in NCBI during 2002–2018 from China. Although different classification methods have been introduced, we still chose the classical classification method because the published F gene sequences were concentrated in region 47–420 nt. From the host perspective, both terrestrial birds and water birds occupied a place at the early phase, but terrestrial birds became the dominant host of class I NDVs after 2014. From the genotype perspective, the sub-genotype 3c has been gradually becoming the predominant genotype since 2010 and is the current epidemic genotype at LBMs [22, 27]. Meanwhile, we found that the occurrences of popular genotypes were not random, and the epidemic genotype would change with the replacement of the host and time.

To further study the sub-genotype 3c, we performed a systematic analysis of sub-genotype 3c NDVs isolated from China. The results showed that terrestrial birds have currently become the dominant host in 3c, and the sub-genotype 3c has constantly been evolving. By plotting the phylogenetic tree of class I NDVs, we were delighted to find two isolates belonging to a new branch. Although these two isolates have been reported to be genotype 3 in NCBI, there is no further sub-genotype classification. Based on the genetic distance of the new sub-genotype classification, the two isolates met the sub-genotype classification condition and were named as the sub-genotype 3d. Furthermore, we analyzed the sub-genotype 3c NDVs during 2009–2018 to confirm whether the 3c underwent amino acid chemotaxis

during evolution. The result revealed that the trend changes of the sub-genotype 3c NDVs existed in the signal peptide of the F protein. The sequence of the signal peptide could guide the membrane fusion of the virus and influence the NDVs [32, 33], which might contribute to the prevalence of the sub-genotype 3c NDVs. It also indicated that the sub-genotype 3c NDV has been evolving to adapt to the environment. Taken the data together, it is necessary to monitor the spread of class I NDVs strictly to prevent the ND outbreak.

Conclusion

Through long-time epidemiological surveillance at LBMs and commercial farms in Eastern China, we gathered data emphasizing that the traditional feeding model was the main cause of class I NDVs circulating at LBMs. The study also indicated the replacement of the epidemic genotype and host in China during 2002–2018, and links were found among the host, genotype, and time. Interestingly, a new sub-genotype of class I was confirmed based on genetic evolution analysis. Furthermore, the epidemic sub-genotype 3c is under ongoing evolution, which might be an adaptive variation to the environment.

Declarations

Acknowledgements

We thank Dr. Xiulong Xu and LetPub (www.letpub.com) for their linguistic assistance during the preparation of this manuscript.

Authors' contributions

XFL and XQW designed the study. XLL carried out the phylogenetic analysis, sequence alignment, and drafted the manuscript. YFS, XW, and LF collected data. NQX and TXL contributed to RNA preparation and RT-PCR. YC and TSZ analyzed the data. MG, JH, ZLH, XWL, and SLH contributed to the revision of the manuscript. All authors approved the content of the manuscript to be published.

Funding

This work was supported by grants from the National Key Research and Development Program of China (2017YFD0500101-3, 2017YFD050080203), by the National Natural Science Foundation of China (31873021), the National Key Technology R&D Program of China (2015BAD12B03). The Earmarked Fund for China Agriculture Research System (CARS-40), the National Key Research and Development Program of China (2017YFD0500702), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

Availability of data and materials

The datasets generated and analyzed during the current study are included in this article and the additional file.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors approved the content of the manuscript to be published.

Competing interests

The authors declared that they had no competing interests.

Author details

¹Animal Infectious Disease Laboratory, College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu, China. ²Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonosis, Yangzhou University, Yangzhou, Jiangsu, China. ³Jiangsu Key Laboratory of Zoonosis, Yangzhou University, Yangzhou, China.

Abbreviations

NDV: Newcastle disease virus; ND: Newcastle disease; LBMs: Live bird markets; RT-PCR: Reverse Transcription-Polymerase Chain Reaction; PCR: Polymerase Chain Reaction; RNA: Ribonucleic Acid; APMV-1: Avian paramyxovirus type 1; ICTV: International Committee on Taxonomy of Viruses; NP: Nucleocapsid protein; P: Phosphoprotein; M: Matrix protein; F: Fusion protein; HN: Hemagglutinin-neuraminidase protein; L: RNA-dependent RNA polymerase protein; NCBI: National Coalition Building Institute; SPF: Specific-pathogen-free; HA: Hemagglutination; HI: Hemagglutination inhibition; BSL3: Biosafety level 3; HEPA: High Efficiency Particulate Air.

References

1. Amarasinghe GK, Ayllon MA, Bao Y, Basler CF, Bavari S, Blasdel KR, Brieseman T, Brown PA, Bukreyev A, Balkema-Buschmann A, et al: **Taxonomy of the order Mononegavirales: update 2019.** *Arch Virol* 2019, **164**:1967-1980.
2. Kim LM, King DJ, Suarez DL, Wong CW, Afonso CL: **Characterization of class I Newcastle disease virus isolates from Hong Kong live bird markets and detection using real-time reverse transcription-PCR.** *J Clin Microbiol* 2007, **45**:1310-1314.
3. Czegledi A, Ujvari D, Somogyi E, Wehmann E, Werner O, Lomniczi B: **Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications.** *Virus Res* 2006, **120**:36-48.
4. Diel DG, da Silva LH, Liu H, Wang Z, Miller PJ, Afonso CL: **Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes.** *Infect Genet Evol* 2012, **12**:1770-1779.
5. Dimitrov KM, Abolnik C, Afonso CL, Albina E, Bahl J, Berg M, Briand FX, Brown IH, Choi KS, Chvala I, et al: **Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus.** *Infect Genet Evol* 2019, **74**:103917.
6. Kim LM, King DJ, Curry PE, Suarez DL, Swayne DE, Stallknecht DE, Slemons RD, Pedersen JC, Senne DA, Winker K, Afonso CL: **Phylogenetic diversity among low-virulence newcastle disease viruses from waterfowl and shorebirds and comparison of genotype distributions to those of poultry-origin isolates.** *J Virol* 2007, **81**:12641-12653.
7. Liu H, Chen FY, Zheng D, Li J, Xu T, Qi L, Wang Z: **Genomic characterization of the first class I Newcastle disease virus isolated from the mainland of China.** *Virus Genes* 2010, **40**:365-371.
8. Wajid A, Rehmani SF, Sharma P, Goraichuk IV, Dimitrov KM, Afonso CL: **Complete Genome Sequence of Genotype VI Newcastle Disease Viruses Isolated from Pigeons in Pakistan.** *Genome Announc* 2016, **4**.
9. Wajid A, Rehmani SF, Wasim M, Basharat A, Bibi T, Arif S, Dimitrov KM, Afonso CL: **Complete Genome Sequence of a Virulent Newcastle Disease Virus Strain Isolated from a Clinically Healthy Duck (*Anas platyrhynchos domesticus*) in Pakistan.** *Genome Announc* 2016, **4**.
10. Habib M, Yaqub T, Nazir J, Shehzad W, Aziz UI R, Sohail T, Mukhtar N, Mehboob A, Munir M, Shabbir MZ: **Habib M, Yaqub T, Nazir J, et al. Genomic and biological characterization of Newcastle disease viruses isolated from migratory mallards (*Anas platyrhynchos*) [J]. Archives of Virology, 2018:1-10. Arch Virol** 2018.
11. Dimitrov KM, Ramey AM, Qiu X, Bahl J, Afonso CL: **Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus).** *Infect Genet Evol* 2016, **39**:22-34.
12. Czegledi A, Wehmann E, Lomniczi B: **On the origins and relationships of Newcastle disease virus vaccine strains Hertfordshire and Mukteswar, and virulent strain Herts'33.** *Avian Pathol* 2003, **32**:271-276.

13. Pedersen K, Marks DR, Arsnoe DM, Afonso CL, Bevins SN, Miller PJ, Randall AR, DeLiberto TJ: **Avian paramyxovirus serotype 1 (Newcastle disease virus), avian influenza virus, and Salmonella spp. in mute swans (*Cygnus olor*) in the Great Lakes region and Atlantic Coast of the United States.** *Avian Dis* 2014, **58**:129-136.
14. Fan S, Wang T, Gao X, Ying Y, Li X, Li Y, Li Y, Ma J, Sun H, Chu D, et al: **Phylogenetic analysis of Newcastle disease viruses isolated from wild birds in the Poyang Lake region of China.** *J Vet Med Sci* 2015, **77**:1143-1149.
15. Liu X, Wang X, Wu S, Hu S, Peng Y, Xue F, Liu X: **Surveillance for avirulent Newcastle disease viruses in domestic ducks (*Anas platyrhynchos* and *Cairina moschata*) at live bird markets in Eastern China and characterization of the viruses isolated.** *Avian Pathol* 2009, **38**:377-391.
16. Alexander DJ, Campbell G, Manvell RJ, Collins MS, Parsons G, McNulty MS: **Characterisation of an antigenically unusual virus responsible for two outbreaks of Newcastle disease in the Republic of Ireland in 1990.** *Veterinary Record* 1992, **130**:65.
17. Shengqing Y, Kishida N, Ito H, Kida H, Otsuki K, Kawaoka Y, Ito T: **Generation of Velogenic Newcastle Disease Viruses from a Nonpathogenic Waterfowl Isolate by Passaging in Chickens.** *Virology* 2002, **301**:206-211.
18. Choi KS, Lee EK, Jeon WJ, Kwon JH, Lee JH, Sung HW: **Molecular epidemiologic investigation of lentogenic Newcastle disease virus from domestic birds at live bird markets in Korea.** *Avian Dis* 2012, **56**:218-223.
19. Zhu J, Xu H, Liu J, Zhao Z, Hu S, Wang X, Liu X: **Surveillance of avirulent Newcastle disease viruses at live bird markets in Eastern China during 2008–2012 reveals a new sub-genotype of class I virus.** *Virology Journal* 2014, **11**:1-9.
20. Wang J, Lv Y, Zhang Y, Zheng D, Zhao Y, Castellan D, Liu H, Wang Z: **Genomic Characterizations of a Newcastle Disease Virus Isolated from Ducks in Live Bird Markets in China.** *PLoS One* 2016, **11**:e0158771.
21. Ren S, Xie X, Wang Y, Tong L, Gao X, Jia Y, Wang H, Fan M, Zhang S, Xiao S, et al: **Molecular characterization of a Class I Newcastle disease virus strain isolated from a pigeon in China.** *Avian Pathol* 2016, **45**:408-417.
22. Zhang P, Xie G, Liu X, Ai L, Chen Y, Meng X, Bi Y, Chen J, Sun Y, Stoeger T, et al: **High Genetic Diversity of Newcastle Disease Virus in Wild and Domestic Birds in Northeastern China from 2013 to 2015 Reveals Potential Epidemic Trends.** *Appl Environ Microbiol* 2015, **82**:1530-1536.
23. Wu S, Wang W, Yao C, Wang X, Hu S, Cao J, Wu Y, Liu W, Liu X: **Genetic diversity of Newcastle disease viruses isolated from domestic poultry species in Eastern China during 2005-2008.** *Arch Virol* 2011, **156**:253-261.
24. Hu S, Ma H, Wu Y, Liu W, Wang X, Liu Y, Liu X: **A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics.** *Vaccine* 2009, **27**:904-910.
25. Hu B, Huang Y, He Y, Xu C, Lu X, Zhang W, Meng B, Yan S, Zhang X: **Avian influenza virus and Newcastle disease virus (NDV) surveillance in commercial breeding farm in China and the**

- characterization of Class I NDV isolates.** *Vet Microbiol* 2010, **144**:82-86.
26. Seal BS, Wise MG, Pedersen JC, Senne DA, Alvarez R, Scott MS, King DJ, Yu Q, Kapczynski DR: **Genomic sequences of low-virulence avian paramyxovirus-1 (Newcastle disease virus) isolates obtained from live-bird markets in North America not related to commonly utilized commercial vaccine strains.** *Vet Microbiol* 2005, **106**:7-16.
 27. Wu W, Liu H, Zhang T, Han Z, Jiang Y, Xu Q, Shao Y, Li H, Kong X, Chen H, Liu S: **Molecular and antigenic characteristics of Newcastle disease virus isolates from domestic ducks in China.** *Infect Genet Evol* 2015, **32**:34-43.
 28. Snoeck CJ, Marinelli M, Charpentier E, Sausy A, Conzemius T, Losch S, Muller CP: **Characterization of newcastle disease viruses in wild and domestic birds in Luxembourg from 2006 to 2008.** *Appl Environ Microbiol* 2013, **79**:639-645.
 29. Miller PJ, Decanini EL, Afonso CL: **Newcastle disease: evolution of genotypes and the related diagnostic challenges.** *Infect Genet Evol* 2010, **10**:26-35.
 30. Carrique-Mas JJ, Trung NV, Hoa NT, Mai HH, Thanh TH, Campbell JI, Wagenaar JA, Hardon A, Hieu TQ, Schultsz C: **Antimicrobial usage in chicken production in the Mekong Delta of Vietnam.** *Zoonoses Public Health* 2015, **62 Suppl 1**:70-78.
 31. Van NTB, Cuong NV, Yen NTP, Nhi NTH, Kiet BT, Hoang NV, Hien VB, Thwaites G, Carrique-Mas JJ, Ribas A: **Characterisation of gastrointestinal helminths and their impact in commercial small-scale chicken flocks in the Mekong Delta of Vietnam.** *Trop Anim Health Prod* 2020, **52**:53-62.
 32. Messina EL, York J, Nunberg JH: **Dissection of the role of the stable signal peptide of the arenavirus envelope glycoprotein in membrane fusion.** *J Virol* 2012, **86**:6138-6145.
 33. York J, Nunberg JH: **Role of the stable signal peptide of Junin arenavirus envelope glycoprotein in pH-dependent membrane fusion.** *J Virol* 2006, **80**:7775-7780.

Tables

Table 1 Information for samples at live bird markets (LBMs) and commercial farms in Eastern China during 2017-2018

Sample type		Collected samples No. (%) ^a	NDV-positive samples	
			No. (%) ^b	
			Class I	Class II
Live poultry markets				
Oropharyngeal and cloacal		1,608 (99.4%)	26 (1.61%)	3 (0.19%)
Environment		10 (0.6%)	0 (0.0%)	0 (0.0%)
Species ^c				
Chickens		639 (39.5%)	23 (1.42%)	3 (0.19%)
Ducks		380 (24.1%)	0 (0.0%)	0 (0.0%)
Geese		582 (36.0%)	3 (0.19%)	0 (0.0%)
Pigeons		7 (0.4%)	0 (0.0%)	0 (0.0%)
Farms (chickens) ^d				
Layers	Farm-A	84(21.0%)	0 (0.0%)	0 (0.0%)
	Farm-B	84(21.0%)	0 (0.0%)	0 (0.0%)
	Farm-C	84(21.0%)	0 (0.0%)	0 (0.0%)
Breeders	Farm-D	74(18.5%)	0 (0.0%)	0 (0.0%)
	Farm-E	74(18.5%)	0 (0.0%)	0 (0.0%)
Farms (ducks) ^e				
Muscovy ducks	Farm-A	200(25.0%)	7(3.5%)	0 (0.0%)
Meat ducks	Farm-B	200(25.0%)	0 (0.0%)	0 (0.0%)
Mixed ducks	Farm-C	200(25.0%)	3 (1.5%)	0 (0.0%)
	Farm-D	200(25.0%)	1 (0.5%)	0 (0.0%)

Newcastle disease virus (NDV)-positive samples by RT-PCR and samples for each flock collected from LBMs and farms in Eastern China during 2017–2018.

^aPercentage of samples collected.

^bPercentage of samples positive for NDV.

^cPercentage of samples collected from each species in live bird markets.

^dPercentage of samples collected from each species in commercial chicken farms.

^ePercentage of samples collected from each species in commercial duck farms.

Table 2 Information on Newcastle disease viruses (NDVs) detected from multiple avian species at live bird markets (LBMs) in Eastern China during 2002–2016

Sample type	Collected samples	NDV-positive samples	
		No. (%) ^a	
		Class I	Class II
<i>Live poultry markets</i>			
Oropharyngeal and cloacal	15,413	308(2.00%)	196(1.27%)
<i>Species (detected)^b</i>	<i>Class I NDV-positive samples</i>		
Chickens		52	
Ducks		45	
Geese		9	

NDV-positive samples for each flock collected from LBMs in Eastern

China during 2002-2016.

^aPercentage of samples positive for NDV.

^bClass I NDVs detected from each species in live bird markets.

Table 3 Estimation of evolutionary divergence among HQ398796, HQ398797, and sub-genotypes of genotype 3 published in NCBI

Sub-genotype ^a	Evolutionary distances ^b				
	HQ398796	HQ398797	3a	3b	3c
HQ398796	0.000				
HQ398797	0.000	0.000			
Sub-genotype 3a	0.044	0.044	0.000		
Sub-genotype 3b	0.071	0.071	0.042	0.000	
Sub-genotype 3c	0.084	0.084	0.045	0.060	0.000

^aAll results were based on the pairwise analysis of 192 nucleotide sequences and the number of sequences analyzed per group were as follows: 3a, n = 14; 3b, n = 71; 3c, n = 105; 3d, n = 2.

^bThe number of base substitutions per site between sequences is shown. Analyses were conducted using the maximum composite likelihood model. The analysis involved 182 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 374 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7.

Table 4 Evolutionary trend of functional domains in F protein of genotype 3c Newcastle disease viruses (NDVs) in China

Functional domain	Fusion protein		
	Signal peptide (1-25)		
Site	8	14	18
2009-2010	S (100.0%)	P (78.3%)	M (53.3%)
		L (15.2%)	V (46.7%)
		S (6.5%)	
2011-2013	S (55.4%)	P (57.1%)	V (96.4%)
	G (42.9%)	L (37.5%)	A (1.7%)
	C (1.7%)	S (5.4%)	L (1.7%)
2014-2018	G (86.8%)	L (93.4%)	V (76.3%)
	S (13.2%)	P (5.3%)	A (23.7%)
		S (1.3%)	
Trend	S→G	P→L	M→V

Sub-genotype 3c viruses (n = 105) were analyzed for amino acids in the key functional regions of the F protein. Protein sequences were translated and analyzed in MEGA 7. According to the variation trend of amino acid sequences in the signal peptide, the time during 2009–2018 was divided into three periods. Other functional regions unlisted did not show an evolutive trend.

Figures

Figure 1

Map of class I Newcastle disease viruses (NDVs) in China during 2002–2018. The green plates indicated that all the class I NDV separation areas in China. The areas marked by the red dot indicated the source of the isolates by our lab mainly in Eastern China. The areas marked by the blue dot indicated the source of other isolates deposited in NCBI. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

Host distribution of class I Newcastle disease viruses (NDVs) in China during 2002–2018. Class I NDV sequences isolated in China were compared according to host species, gray representing water birds and black representing terrestrial birds. Stacked bar charts were drawn by the GraphPad Prism 7.00 software.

Figure 3

Geological distribution of class I Newcastle disease viruses (NDVs) in China during 2002–2018. Class I NDV sequences isolated in China were compared according to genotypes. Stacked bar charts were drawn by the GraphPad Prism 7.00 software. Different genotypes are shown in different colors.

Figure 4

Phylogenetic tree of F partial genes (47-420 nt) of class I Newcastle disease viruses (NDVs) in China during 2002–2018. Only bootstrap values of $\geq 50\%$ are shown. The red sequences are currently popular sub-genotype 3c and the blue sequences are the new sub-genotype 3d. The strains isolated by our laboratory were labeled with ▲. The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 2.48613391 is shown. The percentage of replicate trees

in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 241 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 374 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7.

Figure 5

Distribution of host and time in different genotypes presented as phylogenetic trees in China during 2002–2018. The evolutionary history was inferred using the neighbor-joining method. The optimal tree with the sum of branch length = 2.48613391 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum

composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 241 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 374 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7. Different hosts (A) and periods (B) were distinguished by different colors using the Dendroscope (3.5.7) software.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1.docx](#)