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Characterization of Newcastle disease virus obtained from toco toucan

Jiaxin Li [1,†], **Mengmeng Ling** [1,†], **Yixue Sun** [2,†], **Haiyang Di** [3], **Yulin Cong** [1], **Haiying Yu** [1], **Yanlong Cong** [1,*]

[1]Laboratory of Infectious Diseases, College of Veterinary Medicine, Key Laboratory of Zoonosis Research, Ministry of Education, Jilin University, Changchun 130062, China
[2]Jilin Academy of Animal Husbandry and Veterinary Medicine, Changchun 130062, China
[3]Zoological and Botanical Garden of Changchun, Changchun 130062, China

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***Corresponding author:**
Yanlong Cong
Laboratory of Infectious Diseases, College of Veterinary Medicine, Key Laboratory of Zoonosis Research, Ministry of Education, Jilin University, 5333 Xi'an Road, Lvyuan, Changchun 130062, China.
E-mail: congyanlong@126.com

[†]Jiaxin Li, Mengmeng Ling, and Yixue Sun contributed equally to this work.

ORCID iDs
Jiaxin Li
https://orcid.org/0000-0002-4254-4482
Mengmeng Ling
https://orcid.org/0000-0003-4349-9609
Yixue Sun
https://orcid.org/0000-0001-9351-4147
Haiyang Di
https://orcid.org/0000-0002-3182-7372
Yulin Cong
https://orcid.org/0000-0003-3205-3337
Haiying Yu
https://orcid.org/0000-0003-2721-1484
Yanlong Cong
https://orcid.org/0000-0003-2036-711X

ABSTRACT

Given that the current Newcastle disease virus (NDV) infection in wild birds poses the threat to poultry, surveillance of Newcastle disease in captive wild birds was carried out in Jilin, China in 2018. Here, an NDV strain obtained from toco toucan was firstly characterized. The results showed that the F gene of the NDV isolate Toucan/China/3/2018 is classified as genotype II in class II. Sequence analysis of the F0 cleavage site was ^{113}RQGR/L^{117}, which supports the result of the intracerebral pathogenicity index assay indicating classification of the isolate as low-pathogenicity. Experimental infection demonstrated that Toucan/China/3/2018 can effectively replicate and transmit among chickens. To our knowledge, this is the first report on genetically and pathogenically characterizing NDV strain isolated from toucan, which enriches the epidemiological information of NDV in wild birds.

Keywords: Toucan; NDV; genetic analysis; transmission potential

INTRODUCTION

Newcastle disease (ND) of poultry or ND virus (NDV) infection of wild birds is regarded as a highly contagious disease [1]. It is caused by NDV that is classified as a member of the genus *Othoravulavirus* within a new subfamily *Avulavirinae* of the family *Paramyxoviridae* [2]. In the evolutionary history of the past nearly 100 years, NDV strains are unremittingly evolving with numerous new genotypes and sub-genotypes emerging. Based on the analysis of the full F gene sequence, NDVs are classified into two major groups: class I and class II. Class I contains 1 genotype and class II includes 21 genotypes (I–XXI) [3].

NDV has a wide host range, with at least 250 species in 27 of the 50 orders of birds reported to be capable of natural or experimental infection by NDV [4,5]. Based on the whole information of NDV sequences registered in GenBank from 1930 to April 11, 2019, there are 1,425 strains naturally isolated from at least 169 species of wild birds (data not shown). Among the 17 orders,the most isolates were detected among wild birds in the order *Anseriformes* (n = 649), followed in frequency by the order *Columbiformes* (n = 153), *Galliformes* (n = 139), and *Pelecaniformes* (n = 135) (data not shown). It raises concerns regarding the potential role of wild birds in transmission of NDV. However, epidemiological information about NDV strains circulating in wild birds is still extremely limited.

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Cong Y; Data curation: Li J, Ling M, Sun Y; Investigation: Cong Y, Li J, Sun Y, Di H; Methodology: Li J, Ling M, Sun Y; Resources: Di H; Writing - original draft: Li J, Ling M, Cong Y, Yu H; Writing - review & editing: Cong Y.

Considering the potential presence of NDV in wild birds and the risk that it poses to the poultry industry, an epidemiological investigation of ND in Jilin, China in 2018 was performed, in which captive and free-ranging semidomestic birds, and exotic birds were collected to determine their role in the epizootiology of the disease. As one species of exotic birds, toco toucan (*Ramphastos toco*) (family *Ramphastidae*, order *Piciformes*) is one of the subjects investigated. In 1970s, there is a report of the isolation of NDV strain in toucan [6]. However, the role of this bird in the ecological evolution of NDV is unknown. This report covers detailed documentation of the evolutionary status of an NDV strain obtained from toco toucan and its potential threat to poultry.

MATERIALS AND METHODS

Virus isolation and identification

The initial isolation of NDV was performed in 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs. The allantoic fluid samples were tested using hemagglutination and hemagglutination inhibition assays with standard NDV-specific sera according to the World Organisation for Animal Health (OIE) standard [7]. The NDV isolate was further purified in primary chicken embryo fibroblast cells by plaque assay and then propagated using SPF embryonated chicken eggs. The viral full-length genome sequence was amplified by reverse transcription polymerase chain reaction and sequencing with 11 pairs of primers provided in **Table 1**.

Sequence alignment and construction of phylogenetic tree

Assembly of sequences, translation of nucleotide sequences into amino acid sequences, and initial multiple sequence alignments were performed with the Clustal V method using MegAlign software version 1.03 (DNAStar, USA). Phylogenetic tree from the nucleotide sequences of the full-length F-genes obtained from the GenBank database was constructed

Table 1. Primers used for amplifying the Newcastle disease virus strain of Toucan/China/3/2018 in the study

Name	Primer sequence (5′-3′)	Genomic location
1F	ACCAAACAGAGAATCCG	1–17
1R	GTTGTTGGTGAGCCGC	1,757–1,772
2F	CACATGACCACACCCTC	1,666–1,683
2R	GATTAATTACAGTTGAGCGATC	3,206–3,228
3F	GACCTAAGGTCCAACTC	3,148–3,164
3R	CTAACTGTAAAGAATCAGG	4,451–4,469
4F	TGCGTCTCTGAGATTGCG	4,387–4,404
4R	CGACTGAAGGTAGAGTTACC	5,401–5,420
5F	CAGCAGCGGCTTAATCAC	5,335–5,352
5R	AGCTGACAGACTACCAGAG	6,253–6,271
6F	CACAGATGAGGAACGAAGG	6,207–6,225
6R	CTGAGAACCATACGCGG	7,341–7,357
7F	GGTTGTGATATGCTGTGCTC	7,147–7,166
7R	GCCTTGTATCTCATTGCCAC	8,328–8,347
8F	GTTGCCAGTTGACCACAATC	8,245–8,264
8R	CTTAGCGAAGATCCGTCC	10,031–10,048
9F	GACGACCCTTGAGTACCTTAG	9,955–9,975
9R	CGTGCATAGTCTGCCAGTG	11,724–11,742
10F	TGCGGATAGTCAATTATTCTAG	11,616–11,637
10R	GCACCAATATCTTGCACAG	13,437–13,456
11F	GCTAATCTGTATTACATGTC	13,325–13,344
11R	ACCAAACAAAGATTTGGTG	15,168–15,186

based on 1,000 bootstrap replicates using the maximum-likelihood (ML) approach implemented in MEGA 7.0 software with 1,000 iterations [8]. Genetic distances based on the ML phylogenetic tree were calculated applying Kimura's two-parameter method.

Pathogenicity assessment

To evaluate the pathogenic potential of NDV obtained from toco toucan to chickens, two approaches were used. One is an *in vivo* pathogenicity test to determine the intracerebral pathogenicity index (ICPI) in 1-day-old SPF chicks according to the OIE standard procedures [7]. The other is a molecular pathogenicity approach to analyze the amino acid sequence of the F0 cleavage site.

Cohabitation infection

Two-week-old SPF White Leghorn chickens (n = 18) purchased from Weike Biotechnology(China) were randomly divided into three groups. Group 1 contained 12 chickens, of which 6 chickens were inoculated intranasally with 0.1 mL of $10^{6.0}$ EID_{50}/0.1 mL of allantoic fluid and another 6 chickens were raised together with them for cohabitation infection. Group 2 with 3 chickens was intranasally inoculated with PBS as a negative control. Group 3 with 3 chickens was intranasally inoculated with 0.1 mL of $10^{6.0}$ EID_{50}/0.1 mL of allantoic fluid to be used to analyze the tissues tropism of the virus. All chickens were monitored daily for clinical signs of disease and mortality during the 14-day period. Oropharyngeal and cloacal swabs from each chicken every day were collected and inoculated into the allantoic fluid of 10-day-old SPF embryonated chicken eggs to determine the shedding or transmission potential of the virus. The virus titer was tested using hemagglutination assay according to the OIE standard [7]. The tissue samples from 3 chickens in group 3 at 7 days post inoculation (dpi) were collected and fixed by immersion in 10% neutral buffered formalin for approximately 24 h, then 3 μm sections were prepared for immunohistochemistry (IHC) as previously described [9].

Calculation of antigenic similarity

The antigenic difference between the NDV isolate obtained from toco toucan and vaccine strain LaSota was antigenically analyzed by cross-HI test using the chicken antisera against Toucan/China/3/2018 and LaSota from 14 dpi. The serological relationship was determined by calculating the coefficient of antigenic similarity (R) between each pair of strains. The formula was used: $R = \sqrt{r1 \times r2} \times 100\%$, where γ1 is the ratio of titer of strain A with antiserum B and titer of strain A with antiserum A; γ2 is the ratio of titer of strain B with antiserum A and titer of strain B with antiserum B. When R = 100%, antigenic relatedness is 100%; if R = 50%, antigenic relatedness is 50%; if R = 25%, antigenic relatedness is 25%, etc. In general, R ≤ 25% indicates significant antigenic difference [10].

Ethics statement

This study was conducted in strict accordance with the recommendations in 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 protocols for animal studies were approved by the Committee on the Ethics of Animal Experiments of Jilin University (approval numbers 2018051377–5 for chicken eggs and 2018071599–3 for chickens).

RESULTS

Genomic characteristics

One NDV strain from 1 of 4 toucans in this study was named as Toucan/China/3/2018, the nucleotide sequence of which is available in GenBank under accession number MK902652. The genomic characteristics of Toucan/China/3/2018 were shown in **Table 2**. The lengths of 3′ leader and 5′ trailer are 55 and 114 nt, and the 5′ untranslated regions (UTRs) of six genes are always longer than 3′ UTRs. The gene start sequence of L gene is ACGGGTAGGAC, while that of the other five genes is ACGGGTAGAA. The gene end sequence for NP, M, and L genes is TTAGAAAAAA, while for that of P and HN genes is TAAGAAAAAA, and for that F gene is TTAGAAAAAA. The lengths of intergenic sequence (IGS) of P-M and M-F are 1 nt, while the IGS of NP-P, F-HN, and HN-L are 2 nt, 31 nt, and 47 nt, respectively.

Phylogenetic analysis

Based on the phylogeny of the open reading frame (ORF; 1–1,662 nt) of the F gene, the phylogenetic tree of at least 20 distinct genotypes (I–XXI, except for genotype XV that contains only recombinant sequences) showed that Toucan/China/3/2018 is classified as genotype II (**Fig. 1**). Further analysis displayed that Toucan/China/3/2018 with those strains mainly isolated from pigeons and chickens clustered into a separated clade, which formed a sister relationship with the other cluster where the used vaccine strains, LaSota, Clone 30, B1, and VG/GA, during the past 60 years (**Fig. 2**).

Pathogenicity evaluation

The pathotype of Toucan/China/3/2018 was assessed by two approaches: an *in vivo* pathogenicity test (ICPI assay) and a molecular pathogenicity approach (analysis of amino acid sequence of the F0 cleavage site). The result of the ICPI assay indicated that Toucan/China/3/2018 had an ICPI value of 0.6, which is below the OIE value of equal to or greater than the 0.7 standard for virulent NDV. Thus, the isolate was classified as low-pathogenicity.

Sequence analysis of the F protein gene has been used as an important molecular tool to predict the pathotype of NDV isolates [7]. In the present study, the ORF of the F gene of the isolate Toucan/China/3/2018 contained 1,662 nucleotides, encoding for 553 amino acids. The deduced amino acid sequence of this gene at the cleavage site was ^{113}RQGR/L^{117}, which supports the result of the ICPI assay indicating classification of the isolate as low-pathogenicity.

Transmission potential

To investigate the potential for horizontal transmission of Toucan/China/3/2018, 6 SPF chickens were inoculated intranasally with the virus and another 6 chickens were housed

Table 2. Genomic characteristics of Toucan/China/3/2018

Regions	Gene sequence (nt)	3′UTR (nt)	ORF (nt)	5′UTR (nt)	Intergenic region (nt)	Nucleotide length (nt)	Amino acid length (aa)
Leader	1–55	-	-	-	-	55	-
NP	56–1,801	66	122–1,591	210	2	1,746	489
P	1,804–3,254	83	1,887–3,074	180	1	1,451	395
M	3,256–4,496	34	3,290–4,384	112	1	1,241	364
F	4,498–6,289	46	4,544–6,205	84	31	1,792	553
HN	6,321–8,322	91	6,412–8,130	192	47	2,002	572
L	8,370–15,072	11	8,381–14,995	77	-	6,703	2,204
Trailer	15,073–15,186	-	-	-	-	114	-

UTR, untranslated region; ORF, open reading frame.

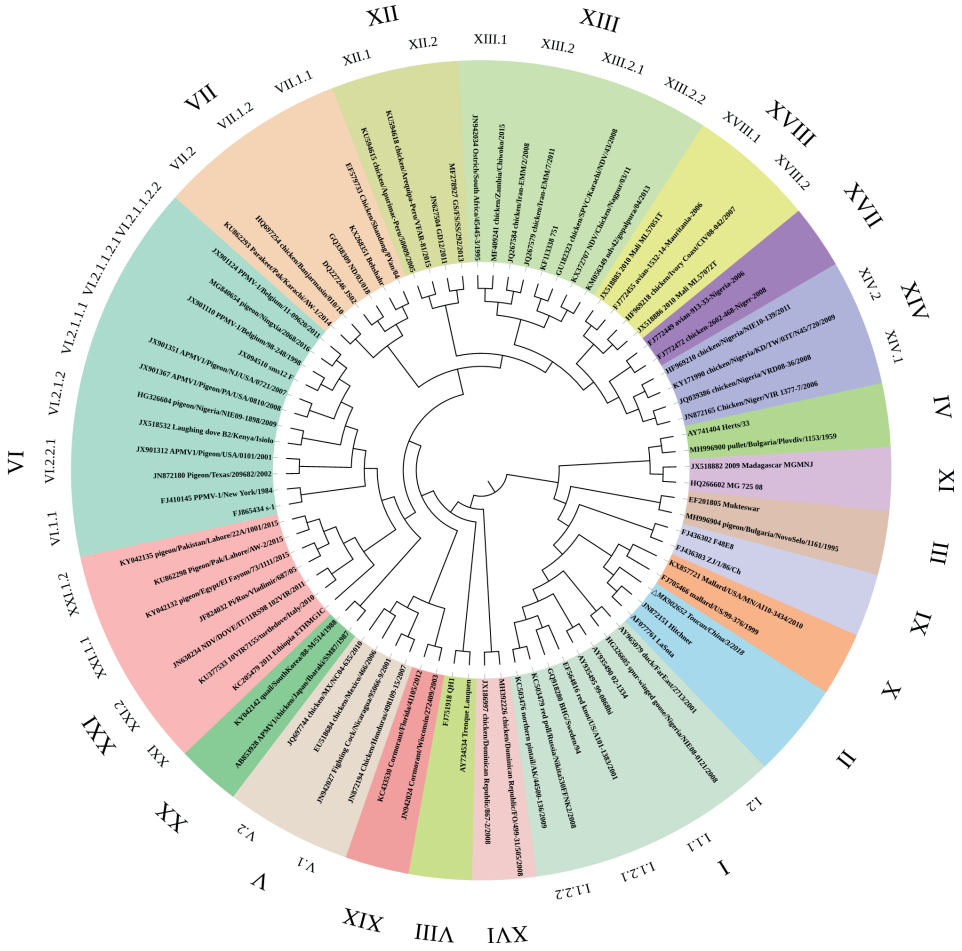

Fig. 1. Phylogenetic analysis based on the open reading frame (1–1,662 nt) of F genes from 20 genotypes of Newcastle disease virus. The tree was created by the maximum-likelihood method and bootstrapped with 1,000 replicates. Toucan isolate analyzed in the present study is in italics.

with them. During the 14-day observation period, the chickens by intranasal inoculation and chickens for cohabitation infection did not show any obvious clinical signs, and none of these chickens died. The supernatant of oropharyngeal and cloacal swabs was inoculated into the allantoic fluid of 10-day-old SPF embryonated chicken eggs. Within 72 h of incubation, no chicken embryos died. After that, allantoic fluid was collected and detected its hemagglutination activity. The result showed that the virus could be detected in swabs from 2 to 14 dpi for the 6 chickens after intranasal inoculation in group 1, while the virus shedding was detected in 1 to 6 of 6 naïve-contact chickens from 4 to 14 dpi in group 1 (**Table 3**).

Tissue tropism

IHC analysis illustrated by **Fig. 3** showed that the chicken by intranasal inoculation at 7 dpi was positive for NDV antigen in almost all tissues and organs such as brain, trachea, lung, heart, liver, spleen, glandular stomach, intestine, pancreas, kidney, thymus, and bursa of Fabricius, indicating that Toucan/China/3/2018 has a broad tissue tropism.

Analysis of antigenic relationship

To elucidate the difference between Toucan/China/3/2018 and LaSota, we analyzed their amino acid sequences and antigenicity. The analysis of the functional domain of the F protein

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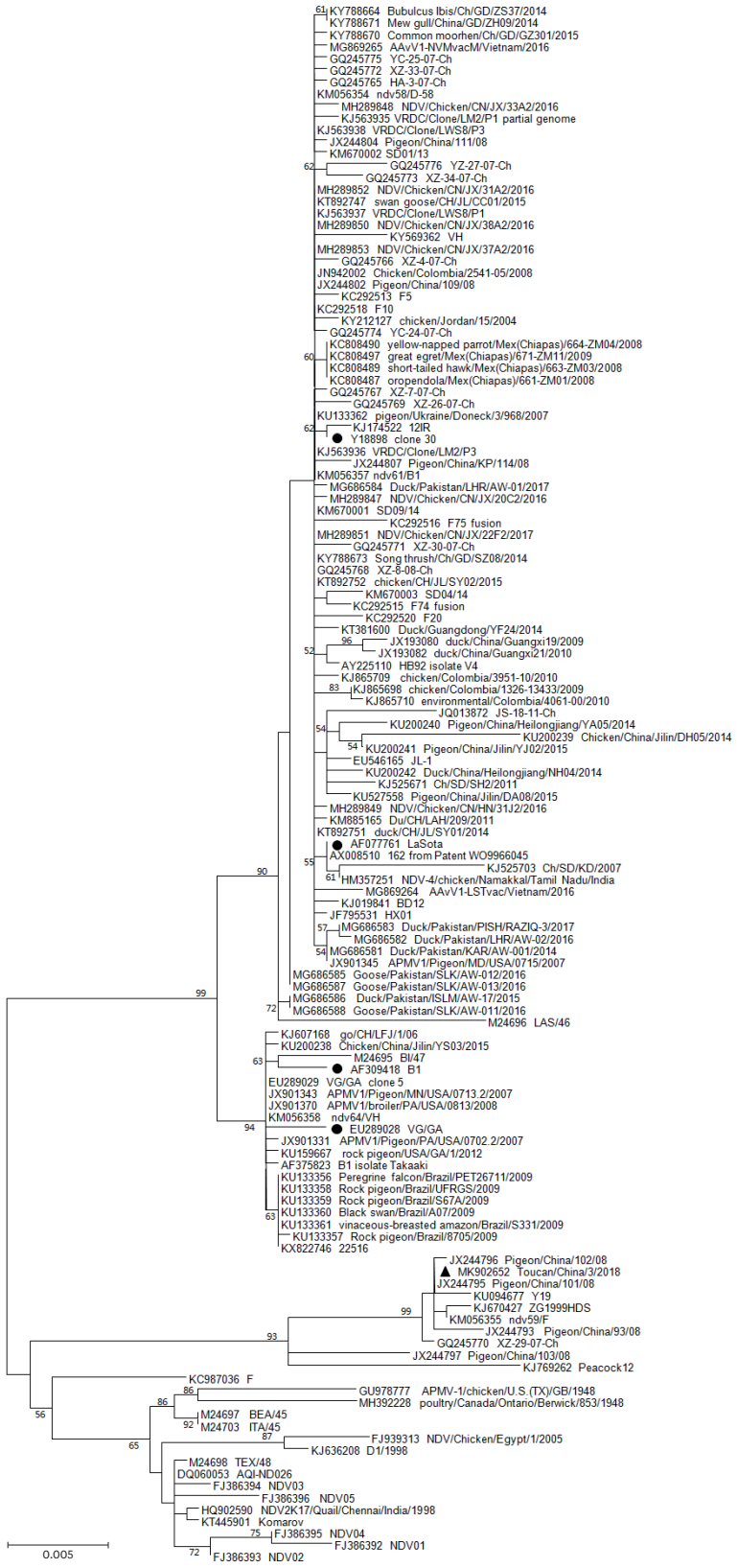

Fig. 2. Phylogenetic analysis based on the full-length (1–1,662 nt) sequence of F genes from genotype II of Newcastle disease virus available in GenBank. The tree was created by the maximum-likelihood method and bootstrapped with 1,000 replicates. Only bootstrap values above 50 are shown. The vaccine strains are shown as solid black circles. Toucan strain analyzed in the present study is shown as a solid black triangle.

Table 3. Frequency of NDV isolation and virus titers from chickens challenged and those for cohabitation infection

Dpi	Chickens							
	Intranasal inoculation				Cohabitation infection			
	O		C		O		C	
	Frequency of NDV isolation	HA titers (nlog2)	Frequency of NDV isolation	HA titers (nlog2)	Frequency of NDV isolation	HA titers (nlog2)	Frequency of NDV isolation	HA titers (nlog2)
1	0/6	-	0/6	-	0/6	-	0/6	-
2	1/6	1.0	0/6	-	0/6	-	0/6	-
3	1/6	1.5	1/6	2.0	0/6	-	0/6	-
4	3/6	2.0	3/6	2.0	1/6	1.5	0/6	-
5	4/6	2.5	5/6	2.0	2/6	2.0	1/6	2.0
6	5/6	4.0	6/6	3.0	4/6	2.0	3/6	2.0
7	5/6	3.5	6/6	2.5	3/6	2.5	5/6	3.0
8	4/6	3.0	6/6	3.0	3/6	2.0	6/6	2.5
10	2/6	2.0	5/6	2.5	2/6	2.0	4/6	3.0
12	1/6	2.5	3/6	3.0	1/6	2.0	3/6	2.5
14	1/6	2.0	2/6	2.5	0/6	-	2/6	3.0

NDV, Newcastle disease virus; dpi, days post inoculation; O, oropharyngeal swab; C, cloacal swab; -, no hemagglutination activity.

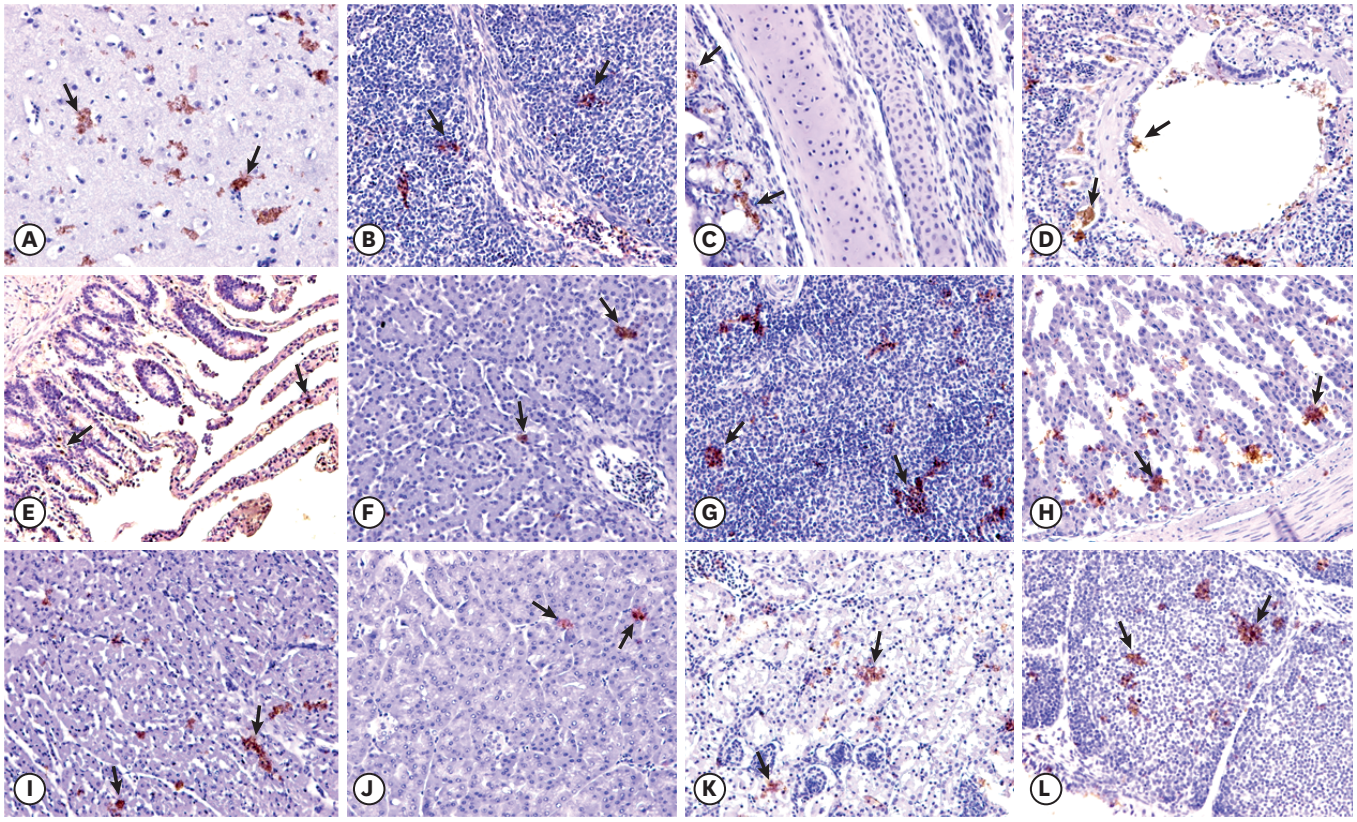

Fig. 3. Immunohistochemistry analysis of tissue samples from chickens intranasally inoculated with Toucan/China/3/2018 at 7 days post inoculation. Arrows indicate the positive labeling of Newcastle disease virus. (A) Brain; (B) thymus; (C) trachea; (D) lung; (E) heart; (F) liver; (G) spleen; (H) glandular stomach; (I) small intestine; (J) pancreas; (K) kidney; and (L) bursa of Fabricius. Arrows indicate magnification, 20×.

showed Toucan/China/3/2018 had only 1 mutation in the heptad repeat C region, and only 1 mutation in the major transmembrane domain, when compared with the consensus amino acid sequence derived from LaSota (**Table 4**). As for the HN comparison, no amino acid substitution was observed at the antigenic sites between them (**Table 5**).

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Table 4. Comparison of amino acid substitutions in the functional domains of the F protein

Strains	HRc (471–500 aa)	Major transmembrane domain (501–521 aa)
	486	520
LaSota	R	I
Toucan/China/3/2018	S	V

HRc, heptad repeat C region.

Table 5. Comparison of amino acid substitutions in the functional domains of the HN protein

Strains	Antigenic sites																		
	1	2				3			4			12		14			23		
	345	513	514	521	569	263	287	321	332	333	356	494	516	347	350	353	193	194	201
LaSota	P	R	I	S	D	N	D	K	G	K	K	G	R	E	Y	R	L	S	H
Toucan/China/3/2018	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Dots indicate residues identical to LaSota.

Table 6. Cross-HI test and coefficients of antigenic relatedness (R%) between Toucan/China/3/2018 and vaccine strain LaSota

Viruses	Antisera		Coefficient of antigenic relatedness
	Toucan/China/3/2018	LaSota	
Toucan/China/3/2018	10,240	10,240	70.71%
LaSota	1,280	2,560	

The antigenic relationship was determined by the cross-HI test (**Table 6**). Using the R value as described above, it was found an R = 70.71% value, demonstrating a definite difference in antigenicity between Toucan/China/3/2018 and LaSota. This finding was in agreement with the result of phylogram F of genotype II (**Fig. 2**).

DISCUSSION

Studies on the diversity, distribution, and potential impact on the poultry industry of NDV circulating in wild birds are extremely limited. As a kind of active and colorful bird, toucans are mainly distributed in tropical forests of South America and become on the verge of extinction. Historically, NDV was isolated from three of 48 toucans in a quarantine station in the United States in the early 1970s [6]. But its genetic background is unclear because of no sequence to follow. In this study, an NDV strain was obtained from toco toucan in China in 2018. To our knowledge, this is the first report on genetically and pathogenically characterizing NDV strain isolated from toucan and its sequence had been registered in GenBank for the first time.

The transmission of NDV occurs through inhalation or ingestion of the virus in aerosol, respiratory discharge and feces from infected birds or contaminated feed, water, equipment and clothing [11]. Animal experiments in this study showed that the virus can be detected in 1 to 6 of 6 chickens for cohabitation infection from 4 to 14 dpi, with the highest rate of virus detection at 8 dpi (**Table 3**). It thus indicated that Toucan/China/3/2018 can transmit between chickens by naïve contact. However, it is unclear how NDV was introduced to toucan. As we know, these investigated toucans had no vaccination history in order to avoid stress. It is necessary to explore whether a vaccine strain spills over from other birds to toucan. As shown in **Fig. 2**, although Toucan/China/3/2018 clustered into a cluster different from that of the common vaccine viruses in the phylogenetic tree of genotype II, it had a close relationship with Pigeon/China/101/08. Thus, it is speculated that the virus is more likely to originate from other wild birds such as pigeons.

From the pathogenic point of view, NDV strains are classified into five pathotypes: asymptomatic enteric, lentogenic, mesogenic, viscerotropic velogenic, and neurotropic velogenic strains [7]. The OIE definition for the virulent NDV is known to be associated with at least three arginine or lysine residues between residues 113 and 116 at the cleavage site at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein [7]. Based on the deduced amino acid sequence of the F gene, Toucan/China/3/2018 has a ^{113}RQGR/L^{117} motif at the cleavage site. Usually, the virulent strain has a ^{113}RQK(R)R/F^{117} motif, whereas ^{113}K(R)QG(E)R/L^{117} residues have been observed in the NDV strains of low-pathogenicity. It thus indicates that Toucan/China/3/2018 is a lowly pathogenic strain. The ICPI test further verified this isolate as low-pathogenicity.

Vaccination is one of the most effective measures in prevention and control of ND outbreak and NDV infection. But it is necessary to make certain whether the most widespread vaccine used in China, LaSota, can protect from Toucan/China/3/2018 infection. The cross-HI assay (**Table 6**) demonstrated that LaSota vaccine is still effective in prevention against the virus isolate, although there is a definite antigenic difference between them. In the future, vaccination against NDV infection in toucans can be considered. Taken together, the NDV isolation from toucan provides valuable information on the epidemiology of ND in wild birds, which emphasizes the need to strengthen routine and import inspection and quarantine measures to prevent ND spread and to further investigate to understand the role of wild birds in the epidemiology of ND.

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