Virus Genes
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Molecular characterization of new emerging sub-genotype VIIh Newcastle disease viruses in China

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Abstract

Newcastle disease (ND) has been enzootic in China for several decades since the first recognition of the disease in 1946 in China. Continuous surveillance revealed that the sub-genotype VIId Newcastle disease virus (NDV) has been predominantly responsible for most of ND outbreak in China in recent years. But in the present study, three virulent NDVs isolated from poultry in southern China were classified as sub-genotype VIIh, which is highly related to the viruses circulating in some Southeast Asia countries. Continuous isolation of genotype VIIh NDV strains in the region suggests its panzootic potential. This is the first report of the sub-genotype VIIh NDVs in domestic poultry in China. The complete genome length of the three isolates was 15,192 nucleotides, and the motif at the cleavage site of F protein was ^{112}RRRRR/F^{117} or ^{112}RRRKR/F^{117}, which was typical of virulent NDV. Phylogenetic analysis based on the F gene revealed that the three viruses had close relationship with the sub-genotype VIIh virus isolated from wild bird in 2011 in China. These viruses might have formed a stable lineage in poultry during 2012–2016 and have the potential to cause enzootic in China. Our study revealed the genetic and phylogenetic characteristics of the three sub-genotype VIIh isolates, which could help us to better understand the epidemiological context of these viruses.

Keywords Newcastle disease virus (NDV) · Sub-genotype VIIh · Genomic characteristics · Phylogenetic analysis

Introduction

Newcastle disease (ND), particularly caused by velogenic strains of Newcastle disease virus (NDV), is one of the most severe diseases of poultry worldwide. NDV is a member of Avian Avulavirus 1 (AAvV-1), which belongs to the genus *Avulavirus* in the family *Paramyxoviridae*. According to their pathogenicity in chickens, the AAvV-1 strains are classified into highly pathogenic (velogenic), intermediate (messogenic), and apathogenic (lentogenic) strains [1]. NDV has a negative sense, single stranded RNA genome with at least three genomic sizes: 15,186, 15,192 and 15,198 nucleotides (nt), and contains six genes, in the order 3′-NP-P-M-F-HN-L-5′, which code for nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin–neuraminidase (HN) and RNA-dependent RNA polymerase (L), respectively [2]. NDVs have been classified into two distinct clades, namely class I and class II, and each class has different genotypes and sub-genotypes [3]. Currently, at least 18 genotypes (I–XVIII) are identified in class II [4].

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Four global panzootics of ND have been confirmed since the first recognition of the disease in 1926. Each panzootic was caused by different genotypes of Newcastle disease viruses [5]. In the past 20 years, genotype VII NDV has been continuously detected from poultry in China and it is now the predominant pathogen responsible for most of outbreaks of ND in China in recent years [6, 7]. However, in recent years, we also detected some new emerging genotypes of NDV in China through active surveillance. In 2010, we first detected the genotype XII NDV in Guangdong, and the source of the virus is still not clear. Genotype VIIh NDVs from poultry has been enzootic in some Southeast Asia countries, such as Indonesia [8], Malaysia [9], Vietnam [10], and Cambodia [11]. In 2011, this sub-genotype VIIh virus was first isolated from wild bird in southern China [12, 13]. During 2012 to 2016, another three sub-genotype VIIh viruses were isolated from domestic poultry in southwestern China, and the pathotype and genotype of the three isolates were characterized to gain the better understanding of the molecular epizootiology in the region.

Materials and methods

Virus isolation and identification

Three NDVs were isolated from tracheal and cloacal swabs collected from asymptomatic poultry from live bird markets (LBMs) of Guizhou province and Yunnan province of China through active surveillance from 2012 to 2016. The isolates were propagated in 9 to 11-day-old specific-pathogen-free (SPF) eggs. The hemagglutination (HA) positive allantoic fluid was collected and identified by standard hemagglutination inhibition (HI) and reverse transcription polymerase chain reaction (RT-PCR) assays. All isolates were plaque purified with three passages on chicken fibroblast (DF1) cells.

Pathogenicity tests

To determine the virulence of the field isolates, the Intracerebral Pathogenicity Index (ICPI) in day-old-chickens was performed according to the procedures described in the OIE Manual of Standards for Diagnostic Tests and Vaccines.

Genome sequencing

The genomic RNA was extracted using High Pure Viral RNA Kit (Roche Applied Science) and amplified by RT-PCR with SuperScript III One-Step RT-PCR Platinum Taq HiFi (Invitrogen). Ten pairs of overlapping specific RT-PCR primers were designed based on sequences of NDV strains which were available in GenBank [14]. For the 3′ leader and 5′ trailer, primers were designed based on specific sequences of the three isolates, and the RNA was amplified using 3′-Full RACE Core Set Ver.2.0 (Takara) and 5′-Full RACE Kit (Takara), respectively, according to the manufacturer's instructions. The amplified products were sequenced at Beijing Genomics Institute, China.

Sequence analyses and phylogenetic studies

Nucleotide sequence editing, analysis and alignments were conducted using the Clustal W multiple alignment method in the MegAlign program of the Lasergene package (DNASTAR Inc., Madison, WI, USA). Phylogenetic tree was constructed by the program MEGA (Version 6.05), using the neighbor-joining method algorithm with 1000 bootstrapped replicates, based on the complete F genes. The sequences used for phylogenetic analysis were downloaded from GenBank.

Results

Virus isolation and identification

Three isolates were identified by HI and RT-PCR assays and designated as chicken/Guizhou/1032/2012, goose/Yunnan/1200/2013 and chicken/Yunnan/1027/2016, respectively. The detailed information of the isolates is shown in Table 1 and Fig. 1.

Pathotyping

The virulence of these isolates was determined by molecular sequencing and ICPI tests. The deduced amino acids at the F0 protein protease cleavage site revealed that the three isolates had motifs that are typical of velogenic strains. One

Table 1 Characteristics of the Newcastle disease viruses characterized in the study

Isolate	Province	Year	Host (scientific name)	MDT	ICPI	Cleavage site of F0
Chicken/Guizhou/1032/2012	Guizhou	2012	Chicken (*Gallus gallus domesticus*)	56	1.838	^{112}RRRRR/F^{117}
Goose/Yunnan/1200/2013	Yunnan	2013	Goose (*Anser domestica*)	56	1.775	^{112}RRRKR/F^{117}
Chicken/Yunnan/1027/2016	Yunnan	2016	Chicken (*Gallus gallus domesticus*)	52	1.875	^{112}RRRKR/F^{117}

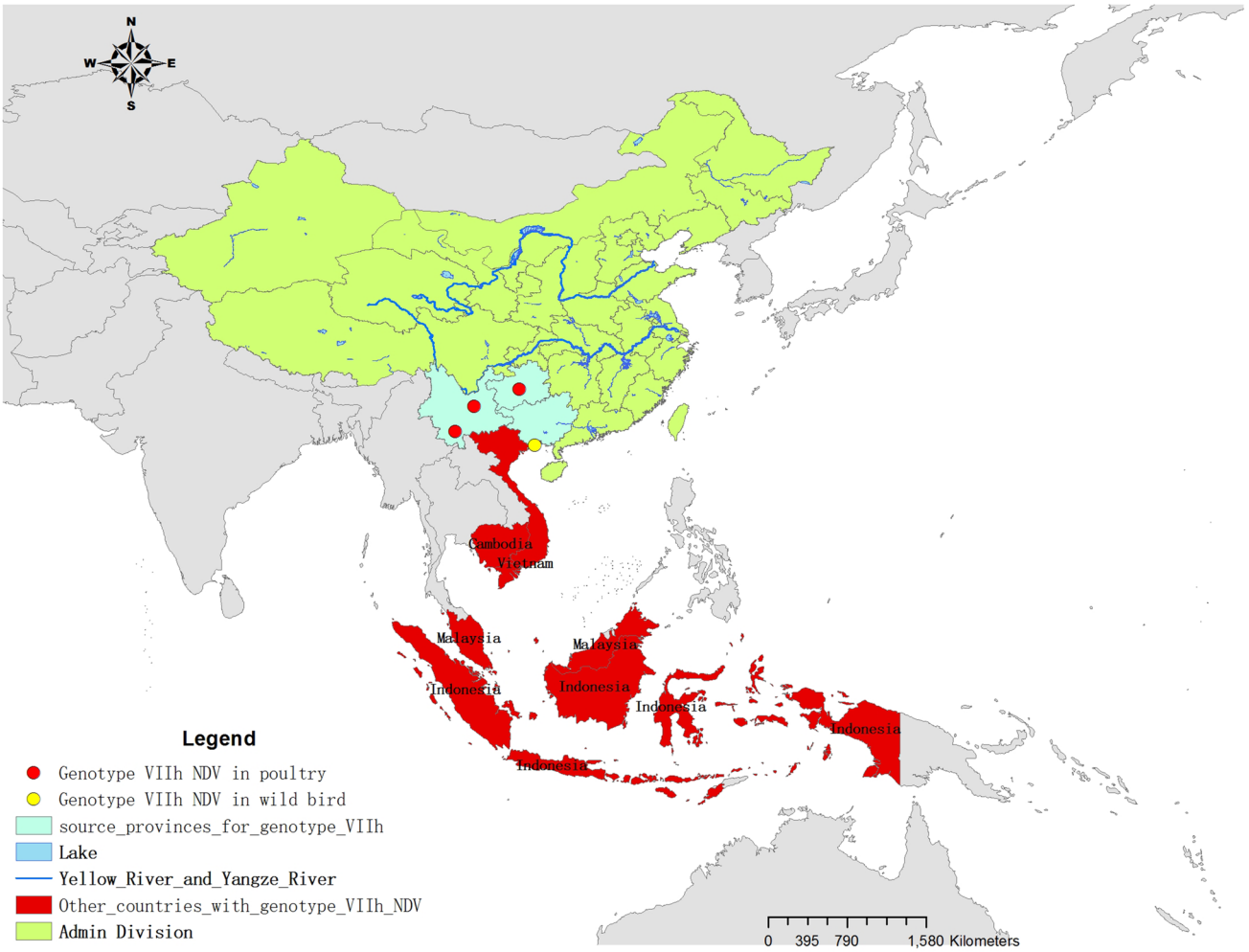

Fig. 1 Distribution of sub-genotype VIIh NDV isolates in Asia

possessed the motif ^{112}RRRRR/F^{117} and the other two had the motif ^{112}RRRKR/F^{117}. As shown in Table 1, the ICPI values were similar for the three cleavage site motifs and confirmed the virulence of these viruses.

Genomic characteristics

The genomic lengths of three isolates in the study were 15,192 nucleotides with the order of 3′-NP-P-M-F-HN-L-5′, and the nucleotide sequence of the three isolates were highly homologous (99.4%). The nucleotide sequence identity of the genome and six genes of three sub-genotype VIIh isolates and eighteen representative NDV strains is shown in Table 2. As shown in Table 3, the 3′ leader and 5′ trailer lengths of the three isolates were 55 and 114 nt respectively as reported for most NDV strains, and the 5′ untranslated regions (UTR) of six genes were longer than 3′ UTRs. The lengths of intergenic sequence (IGS) of NP-P, P-M and M-F were 1 nt, while the IGS lengths of F-HN and HN-L were 31 nt and 47 nt, respectively.

When compared with NDVs belonging to sub-genotypes VIIb, VIId, VIIe, VIIf and VIIg, goose/Yunnan/1200/2013 and chicken/Yunnan/1027/2016 had three amino acid substitutions at the signal peptide (1–31 aa) of F protein, while chicken/Guizhou/1032/2012 had four mutations (Table 4). Moreover, in the heptad repeat regions (HR) of F protein, one substitution D170N in HRa (143–185 aa) was identified in the three viruses, but no mutation was found in HRb (268–299 aa) and HRc (471–500 aa). Six potential glycosylation sites, Asn-X-Ser/Thr (N-X-S/T), were identified in the F protein, which were highly conserved in most NDVs. Seven neutralizing epitopes positioned at residues 72, 74, 75, 78, 79, 157–171, and 343 of the F protein among all the three isolates were conserved. However, analysis of the ten neutralizing epitopes at positions 193–201, 263, 287, 321, 332–333, 346–353, 356, 494, 513–521, and 569 in the HN protein identified one substitution K263R in the three strains.

Table 2 The nucleotide sequence identity of sub-genotype VIIh isolates and other representative NDV strains

Strains	GenBank accession numbers	Genotype	Nucleotide sequence identity (%)						
			Genome	NP	P	M	F	HN	L
Chicken/Guizhou/1032/2012	KT760568	VII	100	100	100	100	100	100	100
Goose/Yunnan/1200/2013	KT760569	VII	99.3	98.9	99.3	99.0	99.4	99.5	99.3
Chicken/Yunnan/1027/2016	KX765879	VII	98.6	98.3	98.0	98.8	99.1	98.5	98.7
Egret/China/Guangxi/2011	JX193074	VII	96.9	98.4	98.5	98.7	97.2	90.4	98.0
Turkey/South_Africa/N2057/2013	KR815908	VII	98.9	98.4	98.6	99.0	98.8	99.2	99.0
I-2	AY935499	I	83.3	81.4	78.5	83.2	84.2	80.5	85.7
La_Sota	AF077761	II	82.8	81.2	79.1	81.7	83.0	78.6	85.6
Mukteswar	EF201805	III	85.2	83.4	81.8	85.0	86.0	81.9	87.5
Herts/33	AY741404	IV	86.7	86.2	84.0	86.4	88.0	83.3	88.3
Anhinga/U.S.(Fl)/44083/93	AY288989	V	85.6	85.5	83.5	84.4	84.8	82.2	87.7
Pigeon/China/SDLC/2011	JQ979176	VI	86.4	86.2	82.5	86.1	86.6	82.8	88.7
QH1	FJ751918	VIII	86.7	86.1	82.9	87.9	87.5	83.0	88.6
F48E8	AF163440	IX	85.0	84.5	81.7	85.0	85.8	80.3	87.3
Mallard/US(MN)/MN00-39/2000	GQ288392	X	83.1	82.0	78.5	82.7	83.3	80.3	85.4
MG_1992	HQ266603	XI	82.8	82.8	80.1	83.3	81.4	79.6	84.9
NDV/peacock/Peru/2011	KR732614	XII	87.9	87.2	84.8	88.4	87.2	85.5	89.8
JKT1997	JX393313	XIII	91.6	91.4	89.6	91.9	91.0	89.2	93.1
Mali_ML029_07	JF966386	XIV	88.6	87.8	86.3	88.3	87.8	86.7	90.2
Chicken/Dominican Republic (Juan-Lopez)/499-31/2008	JX119193	XVI	83.5	81.7	80.1	82.4	83.0	80.4	86.2
Duck/Nigeria/903/KUDU-113/1992	KU058680	XVII	90.9	90.3	88.4	90.7	91.3	88.4	92.5
2009_Mali_ML008	JF966387	XVIII	88.0	87.2	86.0	87.9	88.0	85.1	89.8

Table 3 Genome length characteristics of sub-genotype VIIh isolates

Region	Gene sequence	3′ UTR	Coding sequence	5′ UTR	Intergenic region	Nucleotide length	Amino acid length
Leader	1–55					55	
NP	56–1808	66	122–1591	217	1	1753	489
P	1810–3260	83	1893–3080	180	1	1451	395
M	3262–4502	34	3296–4390	112	1	1241	364
F	4504–6295	46	4550–6211	84	31	1792	553
HN	6327–8328	91	6418–8133	195	47	2002	571
L	8376–15,078	11	8387–15,001	77		6703	2204
Trailer	15,079–15,192					114	

All three isolates used in this study showed the same genome length characteristics

The E347 variation of Hemagglutinin–Neuraminidase linear epitope which was related to antigenic differences was not observed among these isolates in the study [15].

Phylogenetic analysis

Phylogenetic analysis based on sequences of F genes revealed that these isolates belonged to sub-genotype VIIh in class II. All the three VIIh isolates were highly related to one isolate obtained from wild bird in 2011 in Guangxi province from the boarding area (Fig. 2).

Nucleotide sequence accession numbers

The complete genome sequences of the NDV strains characterized in the study have been deposited in GenBank under the accession numbers KT760568 (chicken/Guizhou/1032/2012), KT760569 (goose/Yunnan/1200/2013) and KX765879 (chicken/Yunnan/1027/2016).

Table 4 Amino acid substitutions in the functional domains of the F protein

Strains	Sub-genotype	Signal peptide (1–31 aa)				HRa (143–185 aa)
		9	11	18	25	170
China/Y98/1998	VIIb	I	A	R	C	D
Goode/China/GPV-SF02/2002	VIId	–[a]	–	–	–	–
Fowl/Taiwan/TW-00/2000	VIIe	–	–	–	–	–
Chicken/China/SWS03/2003	VIIf	–	–	–	–	–
China/SRZ03/2003	VIIg	–	–	–	–	N
Chicken/Guizhou/1032/2012	VIIh	T	T	L	F	N
Goose/Yunnan/1200/2013	VIIh	–	T	L	F	N
Chicken/Yunnan/1027/2016	VIIh	–	T	L	F	N

[a]Same amino acid as that in the representative strain of sub-genotype VIIb

Discussion

ND has been enzootic in China for 70 years since the first confirmation of ND in China in 1946. Mass vaccination has been used to prevent the disease since 1980s, and the number of reported outbreaks of ND has declined dramatically in recent years. The results of continuing surveillance indicate that genotype VII NDVs are the predominant pathogens which are responsible for most outbreaks of ND in China in recent years, although viruses of genotypes III and IX have also caused sporadic outbreaks of ND [6, 7, 16]. In recent years, some new emerging NDVs were identified through active surveillance. In 2010, the genotype XII NDV was first isolated from geese in Guangdong in China. This NDV is highly related with one strain isolated from Peru in 2008, however, the source of the virus was not clear. In this study, we have identified three isolates of sub-genotype VIIh from poultry sampled in live bird market through active surveillance in southwestern China, which is the first report of poultry origin VIIh NDVs in China.

The three isolates were identified as virulent strains according to the amino acid residues at F protein cleavage site and ICPI values. The virus chicken/Guizhou/1032/2012 possessed the motif ^{112}RRRRR/F^{117} at the cleavage site of F protein, which was also found in one genotype XI NDV isolated from apparently healthy chicken [17]. Comparison of functional domains of the F and HN proteins between the three sub-genotype VIIh isolates and other viruses belonging to sub-genotypes VIIb, VIId, VIIe, VIIf and VIIg identified several amino acid substitutions. In the F protein, amino acid substitutions were found in the signal peptide and heptad repeat region. As reported, amino acid substitutions in HR region could affect the fusion activity of NDV [18]. In the HN protein, one substitution K263R was identified in the neutralizing epitope. In previous studies, amino acid mutation in neutralizing epitope was reported to result to neutralizing escape variants [19, 20].

The sub-genotype VIIh virus, which has the potential to cause a ND panzootic, has been endemic in some Southeast Asia and Africa countries, such as Indonesia [8], Malaysia [9], Vietnam [10], Cambodia [11], South Africa and Mozambique [21]. In China, the sub-genotype VIIh virus was first isolated from egret in 2011 in Guangxi province [12]. Then three VIIh NDVs were first isolated from domestic poultry in LBMs of two neighboring provinces, Guizhou and Yunan (Fig. 1), from 2012 to 2016 in the study. These isolates were highly related to the virus isolated from wild egret in 2011. Whether there is an epidemiological link between egret and the infected poultry in China need to be further studied. Wild birds have been proven to play an important role in the transmission of highly pathogenetic avian influenza (HPAI) [22, 23]. The role of wild birds in the epidemiology of Newcastle disease has remained less clearly defined either as an incident host or as a potential reservoir. However, wild birds have been implicated as a possible natural reservoir of NDVs since some NDVs have been isolated from wild birds [24–26]. Likewise, the three poultry-origin viruses had a high similarity with the sub-genotype VIIh NDVs circulating in the other Southeast Asia countries. Thus, trading between China and neighboring countries may be another possible cause of the emergence of VIIh NDVs in poultry in China. To clarify the epidemiology of NDVs and reduce the presence of new viruses in China, surveillance of NDVs in wild birds and imported poultry is required.

The three sub-genotype VIIh viruses isolated from 2012 to 2016 had a high nucleotide identity (99.4%) with each other and similar amino acid mutations in the functional domains of F and HN protein, indicating the VIIh NDVs might have formed a stable lineage in some kinds of poultry in China. As reported, the sub-genotype VIIh virus was first isolated from Guangxi province in southern China [13]. Then, in the following years, the virus has transmitted from southern to southwestern China. The sub-genotype VIIh NDVs have been endemic in several countries in Southeast

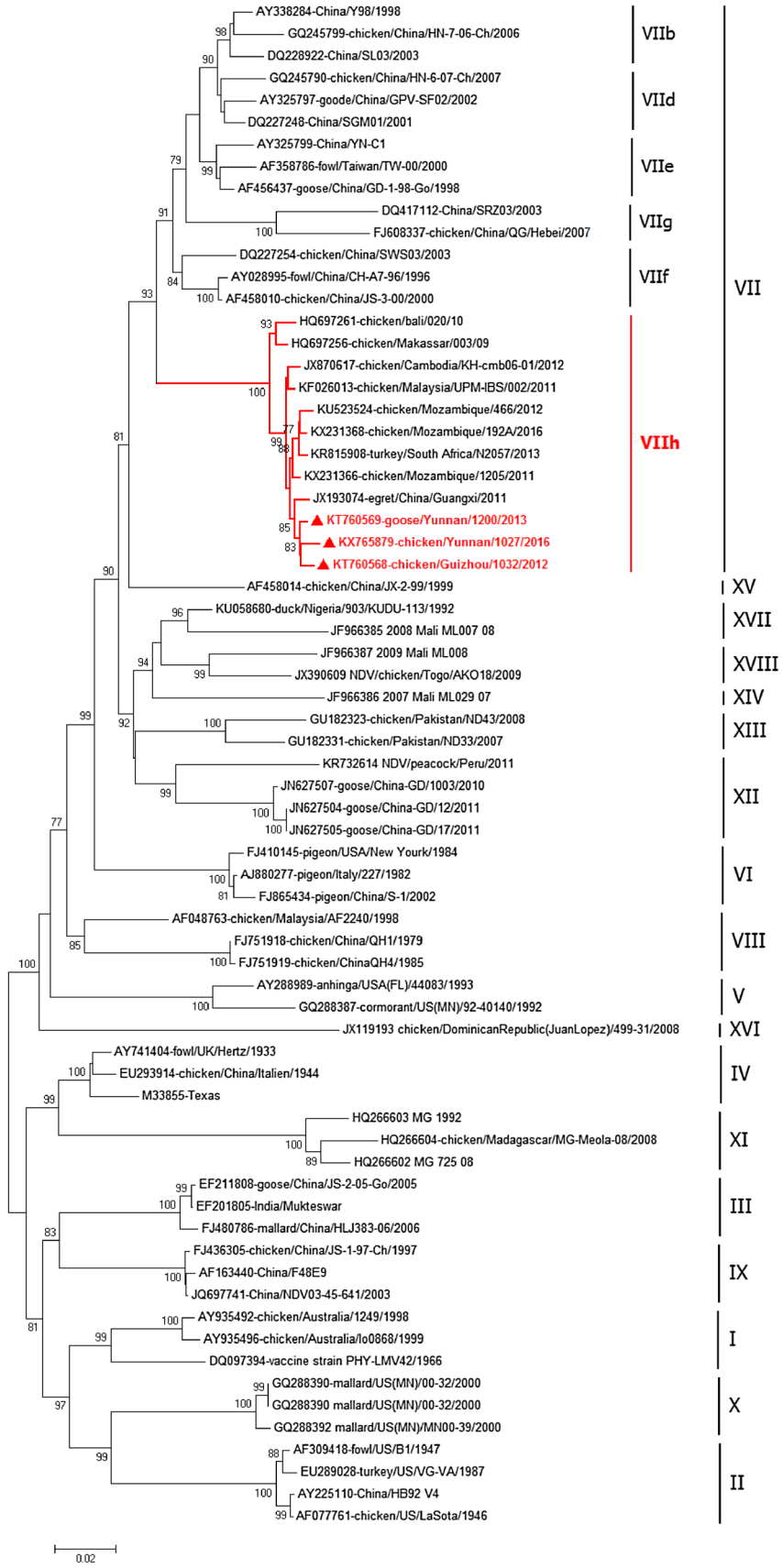

Fig. 2 Phylogenetic tree of subgenotype VIIh NDV isolates based on the complete F gene. The assembly of the matrix sequences was performed using the Clustal W algorithm in MEGA 6.05. The phylogenetic tree was constructed using neighbor-joining method with 1000 bootstrap replicates. The GenBank accession numbers are shown in the tree and the genotype of each strain is indicated at the right. The three strains in this study are marked in red

Asia and Africa, and the similar viruses isolated in China in the recent years also provided an evidence to support the hypothesis that genotype VIIh NDVs had the potential to cause the fifth ND panzootic [12, 21]. The continued spread and transmission of NDVs remain a threat to the domestic poultry industry, so the systematic surveillance of NDVs in China needs to be carried out to clarify the epidemiology and evolution of the sub-genotype VIIh NDVs, which may provide some useful information for prevention and control of this disease.

In conclusion, our study describes the genome characteristics and pathotype of three new emerging sub-genotype VIIh NDVs isolated from domestic poultry, provides important information on the epidemiology of NDV in China with regional implications, and highlights the importance of supporting joint surveillance and disease information sharing in neighboring countries for some transboundary animal diseases, including NDV and HPAI.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures involving animals were approved by the Animal Welfare Committee of China Animal Health and Epidemiology Center (Permit Number: 2014-CAHECAW-06), and conducted according to the guidelines of animal welfare of World Organization for Animal Health.

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