NOTE *Virology*

Phylogenetic analysis of Newcastle disease viruses isolated from wild birds in the Poyang Lake region of China

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ABSTRACT. Newcastle disease virus (NDV) causes a highly contagious viral disease in poultry and wild birds, and it can cause significant economic loss worldwide. Eight viral strains were isolated by inoculating embryonated chicken eggs from the Poyang Lake region of China with swab samples. All eight of the NDV isolates were identified as class I genotype 3 strains, but they diverged notablely from class II viruses. Further analysis revealed that all eight NDV isolates were lentogenic strains containing the ^{112}ERQER↓L^{117} motif at the F protein cleavage site. The strains were highly identical and were more species specific (chicken and waterfowl) than site specific (Nanchang and Duchang regions). The close phylogenetic proximity of these isolates indicates that viral transmission may happen between poultry and wild birds. Our study demonstrates that lentogenic class I NDVs exist in clinically healthy wild waterfowl and poultry within the Poyang Lake region. Active surveillance of these viruses to determine their evolution and origin is one of the most realistic strategies for preventing and controlling NDV outbreaks.

KEY WORDS: genotype, Newcastle disease virus, phylogenetic analysis, Poyang Lake

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Newcastle disease virus (NDV) is a member of the *Avulavirus* genus in the *Paramyxoviridae* family and the *Mononegavirales* order [6]. It causes a highly contagious viral disease in wild birds worldwide, and it can cause severe economic losses in the poultry industry [1, 9, 23]. This non segmented and negative single-stranded RNA virus has a genome of 15,186 to 15,198 nucleotides encoding six major proteins: phosphoprotein (P), nucleoprotein (NP), hemagglutinin-neuraminidase (HN), fusion (F), matrix (M) and RNA polymerase (L) [4]. As the 2 surface glycoproteins, HN and F are the major protective antigens and viral neutralization antigens. The F protein cleavage site sequence is considered to be the major molecular determinant of NDV virulence [11]. Apart from the F protein cleavage site, the HN protein also contributes to NDV virulence [7]. Phylogenetically, NDVs are classified into 2 major groups, class I and class II [5, 12]. Class I viruses encompass at least nine genotypes [17]; however, in a recent study, these have been condensed into a single genotype [8]. These viruses are almost exclusively lentogenic strains and are found in waterfowl and at live bird markets [26]. Class II viruses are generally more virulent and are primarily responsible for the infections observed in pet birds and poultry [3] and are divided into different genotypes [5, 22, 24]. Some studies have indicated that wild birds and waterfowl may play a significant role in the evolution of NDV [16, 18, 21]. However, epidemiological and virological information about NDVs circulation in wild birds and waterfowl is still known little, and their potential to cause disease in domestic poultry is extraordinary limited. Wild aquatic birds are thought to be the natural reservoirs of NDVs of both classes, but they predominantly harbor lentogenic strains [1, 18]. However, some lentogenic strains have the potential to become velogenic after transmission and circulation within poultry populations [27].

Poyang Lake is the largest freshwater lake in China and is an important breeding site for many migratory birds. Thus, there are opportunities for the transmission of viruses among waterfowl, which increases the risk of poultry being exposed to these strains. Here, we report our NDV screening results from wild waterfowl in the Nanchang, Jiujiang and Duchang sites near Poyang Lake. The NDV isolates in this study were characterized by sequencing to determine the genotypes and pathotypes involved.

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Fig. 1. Distribution of collected samples from the Poyang Lake in China. The isolated NDVs are indicated by red "stars".

A total of 284 swab samples were collected from three sites in Jiangxi province (Fig. 1) from March to July of 2013. The collected samples were inoculated into 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs and incubated for 48 hr at 37°C. The presence virus of allantoic fluid was confirmed using the hemagglutination (HA) assay [1] and hemagglutination inhibition (HI) assay with NDV-specific polyclonal antiserum. Of these, eight samples from three bird species (*Anas poecilorhyncha*, *Anser cygnoides* and chicken) contained NDV based on HI analysis. The total isolation rate was 2.8% (4/143 in Nanchang City, 4/119 in Duchang County and 0/22 in Jiujiang County) (Table 2).

To evaluate the pathogenic potential of the isolated viruses, standard assay methods were used to determine the intracerebral pathogenicity index (ICPI) in 1-day-old SPF chicks and the mean death time (MDT) in 9-day-old embryonated SPF chicken eggs [14]. The MDT values of two strains isolated from chicken were 96 hr, however, the MDT values of six strains isolated from wild bird were more than 120 hr. The ICPI values of all 8 strains were 0, indicating that they were all lentogenic. It has been proven that cleavage of the NDV F protein is a major determinant of NDV virulence. The amino acid sequence of the F protein cleavage site is ^{112}R/K-R-Q-R/K-R↓F^{117} for velogenic and mesogenic strains; however, the lentogenic strains F cleavage site is ^{112}G/E-K/R-Q-G/E-R↓L^{117} [7, 17]. In addition, the HN protein also contributes to NDV virulence [7]. To gain a better understanding of NDV virulence in the Poyang Lake samples, the complete F and HN sequences were recovered from the eight isolates. All eight isolates were identified as lentogenic strains containing the^{112}ERQER↓L^{117} motif at the F protein cleavage site.

The coding regions of F and HN genes isolated were used for analysis. Prediction of amino acid sequences and aligment of sequences were performed using MegAlign in the Lasergene V 7.1. The fusion gene was amplified using the following primers: forward primer (47–69): 5′-ATGAATCCCAAGCCCTCTACCAG-3′ and reverse primer (1686–1708):5′-TTACATCTTTGTCGTTGCTCTCA-3′. The HN gene was amplified as described previously [15]. The PCR products were sequenced as described previously [10]. Based on different genotypes, all eight isolates shared unique substitutions (N^{2}-for-D, T^{93}-for-A, D^{104}-for-E, H^{337}-for-Y and N^{489}-for-D) in the F protein sequences compared with other class I viruses (Table 3, other aligned reference sequences are shown in Table S1). Nineteen specific residue substitutions were found in the Class I genotype 3 viruses HN protein sequences compared with other class I viruses (Table 4, other aligned reference sequences are shown in Table S2), and all eight isolates showed unique T^{43}-for-A, S^{60}-for-P/T and S^{577}-for-A substitutions compared with other class I genotype 3 viruses. A comparison of the HN protein sequences between chicken strains (AB859010 and AB859008) and other class I genotype 3 viruses (including 6 wild bird strains isolated) was performed, and the following substitutions E^{293}-for-G, I^{580}-for-T and I^{590}-for-V were found in chicken strains.

The amino acid sequences of the F protein were compared for the 8 isolates, and the sequence identities ranged from 98.5 to 99.5%. The amino acid identities for the 8 isolates

Table 1. Details of F and HN genes used in this study

GenBank accession number	Isolate name	Fusion gene cleavage site (112–117)	Class	Genotype	HN gene	Country
EF564833	Canada goose/US (OH)/87-78/1987	ERQER↓L	I	1	-	USA
HQ008337	Duck/China/JS10/2010	ERQER↓L	I	2	-	China
KF055275	Duck/China/Ch/D10/2009	ERQER↓L	I	2	-	China
JF893453	Duck/China/JX07/2007	ERQER↓L	I	2	-	China
EU493454	Pochard/Finland/13193/2006	ERQER↓L	I	2	-	Finland
HQ398788	Duck/China/NDV09-014/2009	ERQER↓L	I	3	-	China
HQ398789	Duck/China/NDV09-015/2009	ERQER↓L	I	3	-	China
HQ398790	Duck/China/NDV09-016/2009	ERQER↓L	I	3	-	China
FJ597594	Duck/China/D_ZJ_20_05/2005	EQQER↓L	I	3	-	China
FJ597597	Duck/China/ D_SD_29_05/2005	EQQER↓L	I	3	-	China
JN688865	Goose/Jiangsu/ G-JS-09-08/2009	EQQGR↓L	I	3	-	China
FJ597588	Duck/China/D_JS_17_05/2005	EQQER↓L	I	3	-	China
FJ597600	Duck/China/D_ZJ_30_05/2005	EQQER↓L	I	3	-	China
EF564813	Green winged teal/US (AK)/176/1998	ERQER↓L	I	4	-	USA
EF564825	Mallard/US (MD)/02-336/2002	ERQER↓L	I	4	-	USA
AY626267	Duck/U.S./154979-1/2001	ERQER↓L	I	5	-	USA
AY626266	Duck/U.S./119535-1/2001	ERQER↓L	I	6	-	USA
AY626268	Chicken/U.S./101250-2/2001	ERQER↓L	I	6	-	USA
EF564819	Red knot/US (DE)/2026/2000	ERQER↓L	I	7	-	USA
EF564820	Mallard/US (MD)/04-125/2004	ERQER↓L	I	7	-	USA
JN941987	Northern Pintail/Idaho/19663-2/2009	ERQER↓L	I	7	-	USA
EF564815	Dunlin/US (DE)/A100-2093/2000	ERQER↓L	I	8	-	USA
EF564831	Ruddy turnstone/US (DE)/401/2004	ERQER↓L	I	8	-	USA
EF565029	Blue winged teal/US (TX)/02-11/2002	ERQER↓L	I	9	-	USA
AB858995*	Anser cygnoides/Duchang/J17-13-F/2013	ERQER↓L	I	3	-	China
AB858996	Anas poecilorhyncha/Nanchang/J72-13-F/2013	ERQER↓L	I	3	-	China
AB859001	Anas poecilorhyncha/Nanchang/J70-13-F/2013	ERQER↓L	I	3	-	China
AB859002	Chicken/Nanchang/J2-13-F/2013	ERQER↓L	I	3	-	China
AB858997	Anser cygnoides/Duchang/J55-13-F/2013	ERQER↓L	I	3	-	China
AB858998	Anas poecilorhyncha/Duchang/J77-13-F/2013	ERQER↓L	I	3	-	China
AB858999	Anas poecilorhyncha/Duchang/J80-13-F/2013	ERQER↓L	I	3	-	China
AB859000	Chicken/Nanchang/J36-13-F/2013	ERQER↓L	I	3	-	China
FJ597604	Duck/China/D/AH/6/04/2004	EKQGR↓L	II	Ib	-	China
FJ597609	Duck/China/D/JS/51/05/2005	GKQGR↓V	II	Ib	-	China
DQ195265	USA/LaSota/2005	GRQGR↓L	II	II	-	USA
JN618349	Chicken/China/JS-3-05-Ch/2005	RRQKR↓F	II	VII	-	China
DQ659677	China/NA-1/2006	RRQKR↓F	II	VII	-	China
NC 005036	Goose/China/SF02/2002	RRQKR↓F	II	VII	-	China
AB859003	Anser cygnoides/Duchang/J17-13-HN/2013	-	I	3	HN	China
AB859004	Anas poecilorhyncha/Nanchang/J72-13-HN/2013	-	I	3	HN	China
AB859005	Anser cygnoides/Duchang/J55-13-HN/2013	-	I	3	HN	China
AB859006	Anas poecilorhyncha/Duchang/J77-13-HN/2013	-	I	3	HN	China
AB859007	Anas poecilorhyncha/Duchang/J80-13-HN/2013	-	I	3	HN	China
AB859008	Chicken/Nanchang/J36-13-HN/2013	-	I	3	HN	China
AB859009	Anas poecilorhyncha/Nanchang/J70-13-HN/2013	-	I	3	HN	China
AB859010	Chicken/Nanchang/J2-13-HN/2013	-	I	3	HN	China
HQ997403	Chicken/China/NDV10-059/2010	-	I	3	HN	China
HQ997404	Chicken/China/NDV10-060/2010	-	I	3	HN	China
HQ997405	Chicken/China/NDV10-061/2010	-	I	3	HN	China
HQ997406	Chicken/China/NDV10-062/2010	-	I	3	HN	China
HQ997410	Chicken/China/NDV10-069/2010	-	I	3	HN	China
HQ398840	Chicken/China/NDV09-043/2009	-	I	3	HN	China
HQ398841	Chicken/China/NDV09-044/2009	-	I	3	HN	China
HQ398846	Chicken/China/NDV09-051/2009	-	I	3	HN	China
HQ398847	Chicken/China/NDV09-052/2009	-	I	3	HN	China
HQ398848	Chicken/China/NDV09-053/2009	-	I	3	HN	China
HQ398849	Chicken/China/NDV09-054/2009	-	I	3	HN	China

"*"The bold indicated NDV strains isolated in this study

Table 2. Samples collected and NDV strains isolated in this study

Location	Species	No. of samples		No. of positive (%)	Class (genotypes)
		Oral	Cloacal		
Nanchang	*Anas poecilorhyncha*	100	0	2 (2.0)	I (3)
	Chicken	0	43	2 (4.7)	I (3)
Jiujiang	*Anser cygnoides*	22	0	0	
Duchang	*Anas poecilorhyncha*	43	25	2 (2.9)	I (3)
	Anser cygnoides	37	14	2 (3.9)	I (3)
Total		202	82	8 (2.8)	

Table 3. Specific amino acids substitution in the fusion protein sequences in this study

GenBank accession number	Class	Genotype	Consensus amino acids and its position in the fusion protein				
			^{2}D	^{93}A	^{104}E	^{337}Y	^{489}D
AB524405	I	1	.	.	.	.	.
JF893453	I	2	.	.	.	.	.
FJ597594	I	3	.	.	.	.	.
FJ597597	I	3	.	.	.	.	.
EF564825	I	4	.	.	.	.	.
AY626267	I	5	.	.	.	.	.
AY626266	I	6	.	.	.	.	.
EF564819	I	7	.	.	.	.	.
EF564815	I	8	.	.	.	.	.
AB858995-9002	I	3	N	T	D	H	N
NC 005036	II	VII	G	T	G	.	E
FJ597609	II	Ib	G	T	.	.	.
DQ195265	II	IV	G	T	.	.	.

Amino acids that match the consensus exactly are denoted by '·'

Table 4. Specific amino acids substitution for HN protein in this study

GenBank accession number	Consensus amino acids and its position in the HN protein of Class I genotype 3																		
	^{7}Q	^{43}A	^{52}T	^{60}P/T	^{61}V	^{178}F	^{248}D	^{261}D	^{293}G	^{308}I	^{311}R	^{381}T	^{396}T	^{422}S	^{423}P	^{567}R/Q	^{577}A	^{580}T	^{590}V
AB859003	.	T	.	S	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.
AB859005	E	T	S	S	E	.	H	.	.	.	.	.	.	C	.	.	S	.	.
AB859006	.	T	.	S	.	.	.	.	.	K	.	.	.	.	.	.	S	.	.
AB859007	.	T	.	S	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.
AB859004	.	T	.	S	.	.	.	.	.	.	P	.	.	.	.	.	S	.	.
AB859009	.	T	.	S	.	L	.	H	.	.	.	S	.	.	.	.	S	.	.
AB859010 (chicken strain)	.	T	.	S	.	.	.	.	E	.	.	.	N	.	A	W	S	I	I
AB859008 (chicken strain)	.	T	.	S	.	.	.	.	E	.	.	.	.	.	.	.	S	I	I

Amino acids that match the consensus exactly are denoted by '·'

differed from other class I viruses (genotypes 1 to 9, presented in Table 1) by 3.3 to 10.8%, whereas the isolates differed from class II (presented in Table 1) viruses by 22.7 to 33.2%. The HN coding regions of the 8 isolates were 1,851 nt in length and encoded a protein product consisting of 617 amino acids. The amino acid identities for the HN regions from the isolates were compared to other class I genotype 3 viruses (presented in Table 1), and the isolates differed from the other viruses by 0.9 to 7.3%.

To estimate the risk of the viruses pose to poultry populations, it is essential to evaluate the evolution of these viruses. Prior to phylogenetic analysis, ClustalX 2.0 [19] and Lasergene V 7.1 software packages were used for sequences analysis. MEGA 5.0 software was used for phylogenetic analysis using a neighbor-joining method with 1,000 bootstrap replicates [28]. The strains of F gene are shown in Table 1. Phylogenetic analyses using the 1,662-bp region of the F gene and the 2001-bp region of the HN gene sequence indicate that all eight NDV strains form a distinct cluster within the genotype 3 viruses and are most closely related to Chinese poultry isolates. A phylogenetic analysis of F gene revealed that the isolated strains were highly identical and were more species

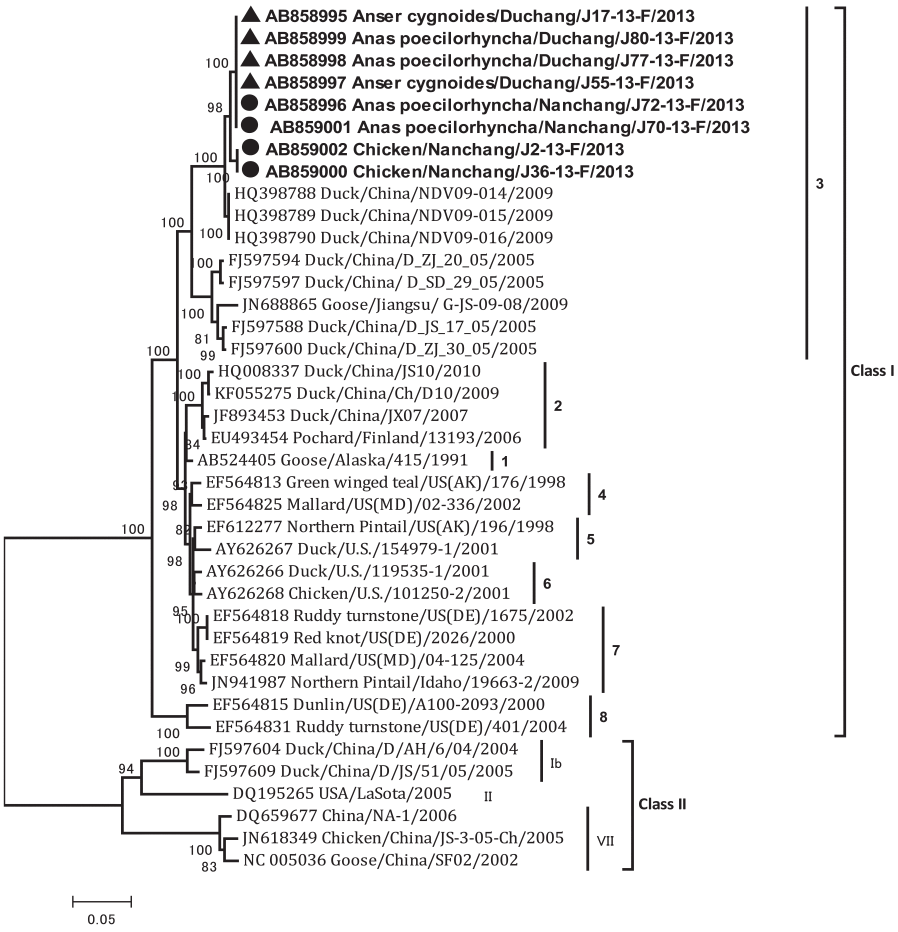

Fig. 2. Phylogenetic analysis of the nucleotide sequences of the NDV F gene (nt 1–1,662).The sequences in our study are indicated by circles (Nanchang strains) and triangles (Duchang strains). Bootstrap values (based on 1,000 replicates) for each node are provided if >75% of the values were available.

specific (chicken and waterfowl) than site specific (Nanchang and Duchang regions). Two branches radiate out from class I genotype 3 (Fig. 2). The first branch contains poultry viruses, all of which originated in eastern China and were isolated between 2005 (D/ZJ/20/05, D/SD/29/05, D/JS/17/05 and D/ZJ/30/05) and 2009 (G-JS-09-08). The other cluster includes viruses associated with poultry viruses isolated in eastern China in 2009 (NDV09-014, NDV09-015 and NDV09-016) and isolated from *Anas poecilorhyncha, Anser cygnoides*, and chickens in our study. *Anas poecilorhyncha* and *Anser cygnoides* are found in most freshwater habitats, which are widespread throughout China. This finding indicates that class I genotype 3 viruses have also emerged in the wild bird and poultry population in China. A phylogenetic comparison of HN gene sequences from all eight NDV isolates and 11 reference NDVs from GenBank (all class I genotype 3) demonstrated that the 8 NDV strains form a distinct cluster within the genotype 3 viruses and are different from strains isolated between 2009 and 2010 (Fig. 3).

Most poultry in the Poyang Lake region are raised in a free-range style. This raises the possibility that migratory birds could introduce viruses into the local resident wild bird population, which could then distribute the virus to local domestic birds. Migratory wild birds have been shown to transmit NDVs to free-range poultry through direct contact or through the contamination of water or feed sources [29]. In our study, 2 NDVs isolated from chickens indicate that domestic poultry would shed these lentogenic viruses, which may be prevalent in local poultry populations. The close phylogenetic proximity of these isolates indicates that viral transmission may occur between poultry and wild birds. Efforts are needed to restrict the interactions between wild birds and poultry, as these 2 hosts appear to be subject to continuing exchange of NDV strains [20].

Since the discovery of NDV in 1926, ND has been regarded as endemic to many countries. Vaccination has been widely used for many years to prevent and control ND in China, but this disease is still sporadic and is a fatal viral disease for poultry industry [25]. Most NDV research has been performed on virulent isolates, and very little is known about

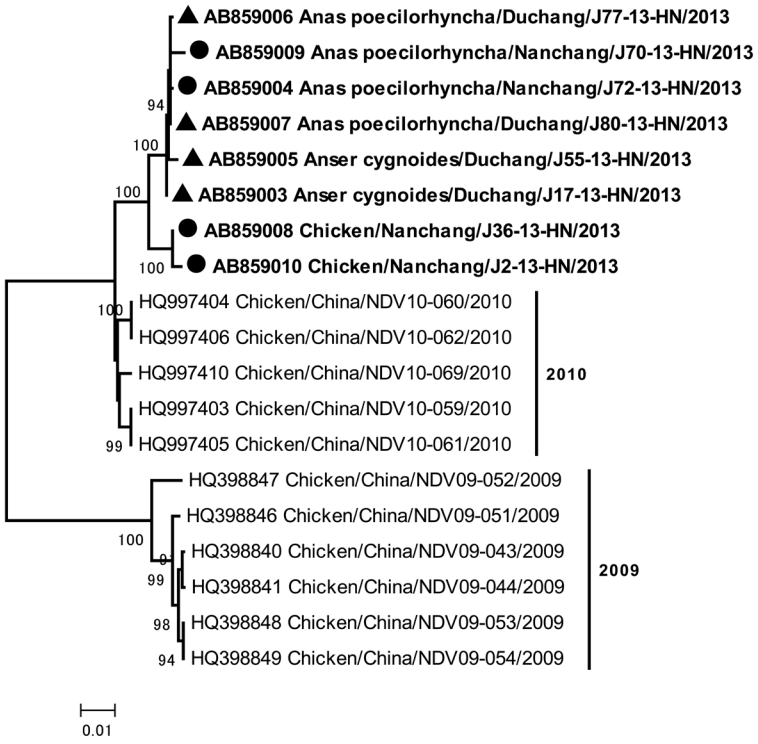

Fig. 3. Phylogenetic analysis of the nucleotide sequences of class I genotype 3 HN gene sequences (nt 1–2,001). The sequences in our study are indicated by circles (Nanchang strains) and triangles (Duchang strains). Bootstrap values (based on 1,000 replicates) for each node are provided if >75% of the values were available.

the evolution of lentogenic NDVs [23]. Wild aquatic birds are the natural reservoirs of NDV, and they generally harbor lentogenic strains [15], but lentogenic strains have also been detected in poultry vaccinated with live virus. However, lentogenic viruses would have the potential to increase their virulence long time or from one host to another [2, 27]. The MDT values of strains isolated from chickens are lower than those of strains isolated from wild birds. In addition, 3 residue substitutions were found in the HN protein sequences of strains isolated from chickens compared to strains isolated from wild birds. These results indicated that the MDT values and the mutation of the NDVs are related to the host. Moreover, the new host environment may play a selective forces role in the acquisition of virulence [30]. In Australia, endemic lentogenic viruses have been circulating in domestic poultry for more than 30 years, but unknown conditions have since caused these NDVs to undergo genomic changes, resulting in a more virulent genotype [13]. These results indicate that lentogenic poultry strains may exist in nature through waterfowl-to-domestic poultry transmission.

GenBank accession numbers: All the sequences (n=16) used in this study were submitted to GenBank with F gene accession numbers (AB858995-AB859002) and HN gene accession numbers (AB859003-AB859010).

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