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LETTER TO THE EDITOR

Molecular characteristics of a canine distemper virus isolated from mink (*Neovison vison*) in Shandong, China, 2013

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Canine distemper (CD) is a highly contagious disease that affects a wide variety of domestic and wild carnivores (*1*). Clinical symptoms in CD-infected animals depend on strain virulence, environmental condition, species identity, host age and immune status. Typical signs include vomiting, diarrhea, dehydration, excessive salivation, coughing and/or labored breathing, loss of appetite, and weight loss. The respiratory, gastrointestinal, integumentary and central nervous systems are most commonly affected in all species (*2*). The etiological agent of CD is canine distemper virus (CDV), which has been renamed canine morbillivirus, according to the latest virus taxonomy of the International Committee on Taxonomy of Viruses. CDV is classified into the genus *Morbillivirus* in the family *Paramyxoviridae*, and its genome is a single strand of RNA with negative polarity, which encodes six structural (N, P, M, F, H and L) and two nonstructural (V and C) proteins in the order of 3'-N-P (V/C)-M-F-H-L-5'.

The CDV H protein, a major determinant of host tropism and cytopathogenicity (*3*), helps in viral attachment to its host cell through interaction with a cellular receptor. The CDV H gene shows a great genetic variation among the six structural genes (*4–5*), and therefore is used for determining CDV genetic lineages, which generally correspond to viral geographic origins. There are currently at least seventeen major genetic lineages, namely the America-1 to -5, Europe wild-life, Arctic, South Africa, Europe-1/South America-1, South America-1 to -3, Rockborn-like and Asia-1 to -4 (*6*), whereas only one serotype has been reported worldwide. An H gene-based phylogenetic analysis shows that the majority of CDVs in China belong to the Asia-1 lineage (*7*), which also contains other CDVs isolated from Japan, Chinese Taipei and South Korea (*8-10*).

Live-attenuated vaccines have been widely used in susceptible animals, such as dog, mink (*Neovison vison*) and fox, while CD infection is still frequently reported in China. In October 2013, severe CD infection was identified at a mink farm in Zhucheng City of Shandong Province, China. Clinical signs in diseased minks were characterized by eye and nose discharge, diarrhea, loss of appetite, labored breathing, but no neurological symptoms. This CD infection was subsequently confirmed in our laboratory. A CDV strain was isolated from a dead mink, and then subjected to seven serial passages in Vero-SLAM cells. In the present study, this CDV isolate (ZC1310M strain) was characterized by next-generation sequencing for further analyzing its genome, especially the H gene.

Total RNA was extracted from the culture supernatant of passage-7 CDV using TRIzol™ Reagent (Invitrogen™) according to the manufacturer's instruction, and then subjected to the next-generation sequencing at the Shanghai Tanpu Biotechnology Co., Ltd, China. *De novo* assembly

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Abbreviations: CD = canine distemper; CDV = CD virus; ORF = open reading frame

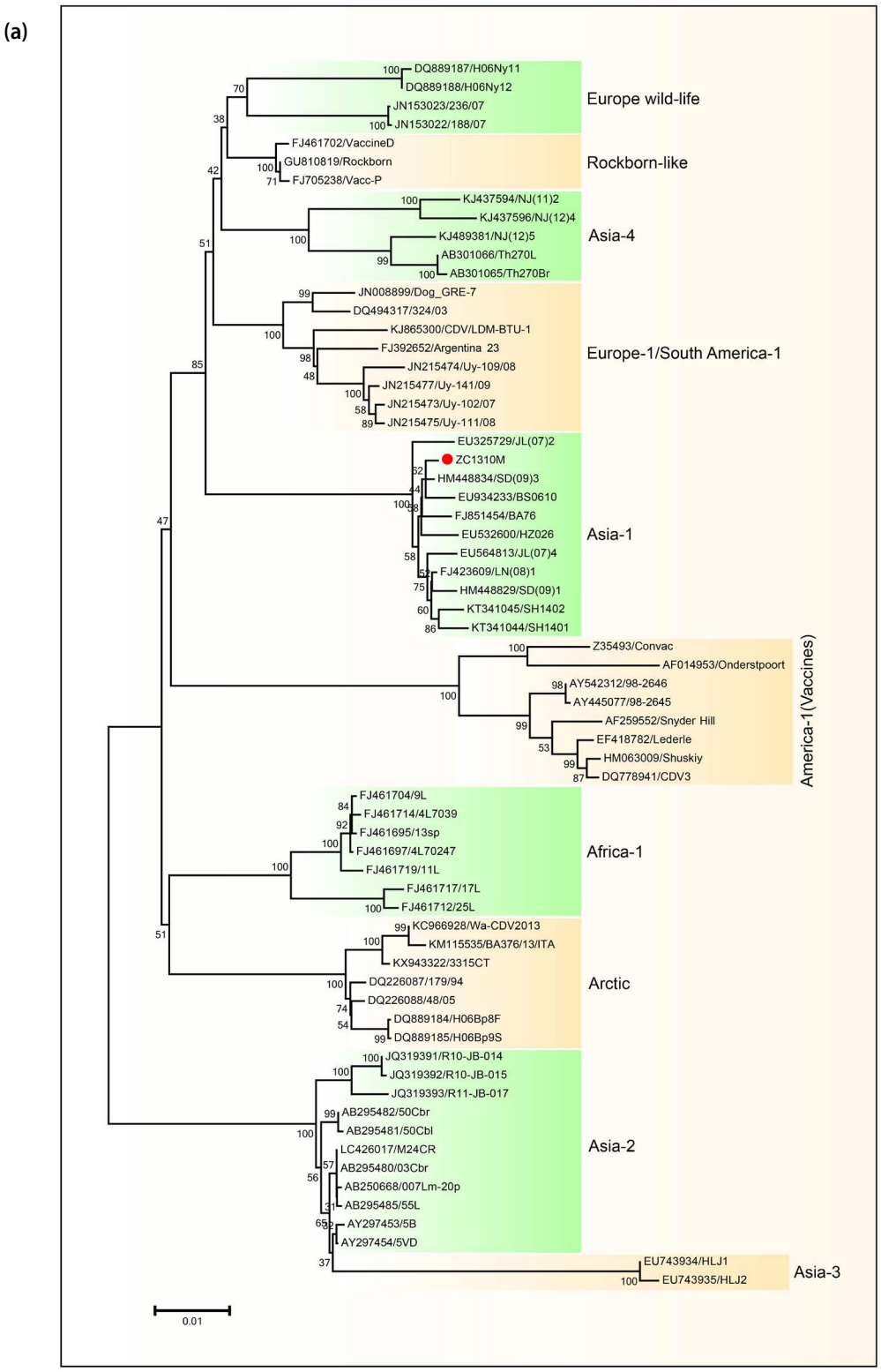
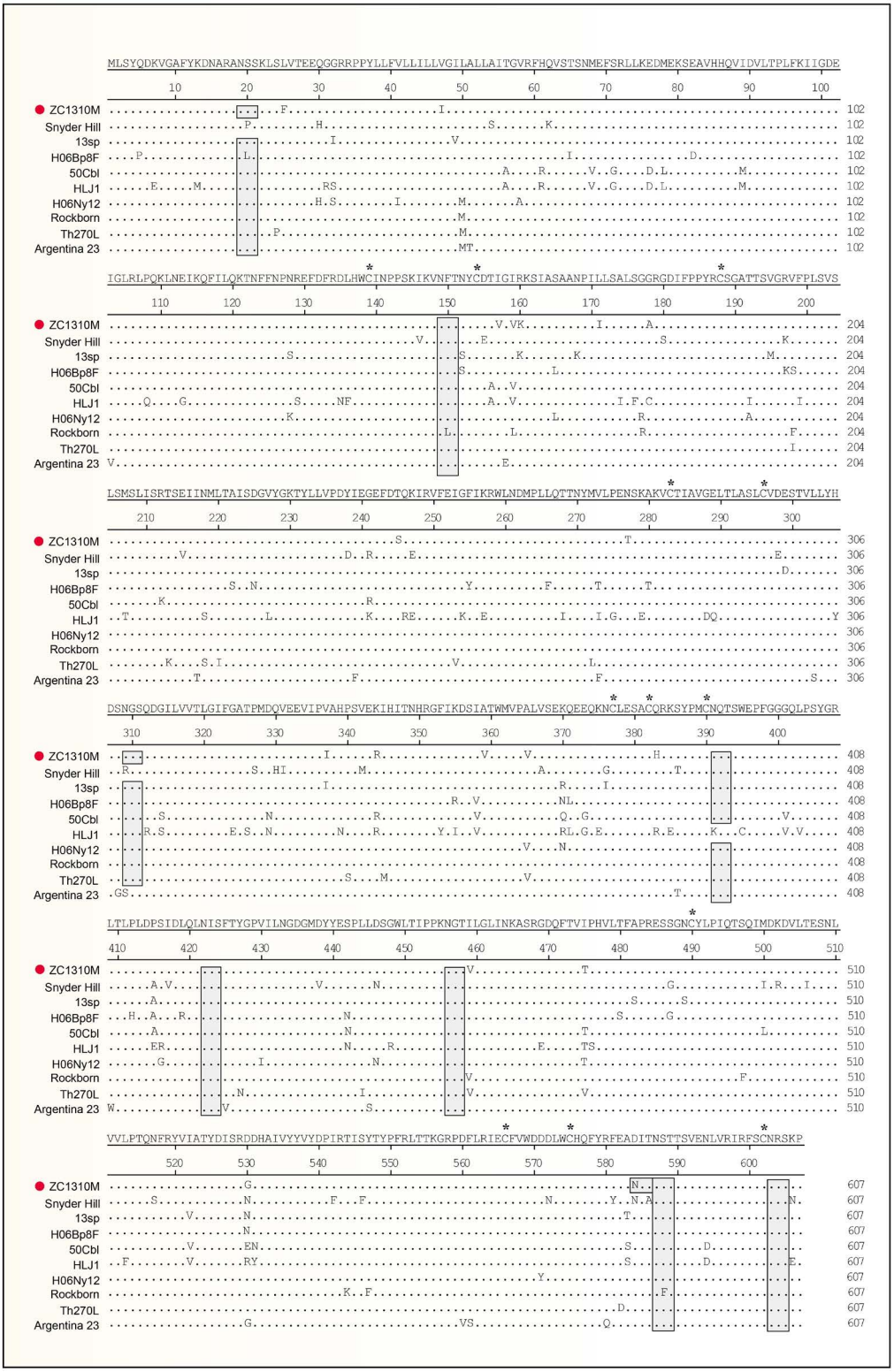


Fig. 1

Phylogenetic relationship among the ZC1310M and representative strains in the GenBank by alignment of their H ORFs (a) and alignment of deduced aa sequences for CDV H proteins (b)

(a) The Neighbor-joining method is used to construct the phylogenetic tree using MEGA 7.0 software. Numbers above and below branches indicate bootstrap values (1000 replicates). The phylogenetic tree includes ten genetic lineages, namely the Europe wild-life, Rockborn-like,



Asia-4, Europe-1/South America-1, Asia-1, America-1 (Vaccines), Africa-1, Arctic, Asia-2 and Asia-3. The "ZC1310M" (GenBank: MN326827) is marked with a red circle, and "GenBank Acc. No./strain name" is quoted for other strains. **(b)** Ten representative strains (ZC1310M, Snyder Hill, 13sp, H06Bp8F, 50Cbl, HLJ1, H06Ny12, Rockborn, Th270L and Argentina 23) are accordingly selected from the ten genetic lineages. The "ZC1310M" is marked with a red circle. The potential N-linked glycosylation sites are boxed. Dots and asterisks indicate identical amino acids and conserved cysteine residues, respectively.

of the raw sequencing data was completed by SPAdes as described previously (*11*). The complete sequence of ZC1310M genome was 15,690 nucleotides (42.68% C+G), in agreement with the "rule of six" of paramyxoviral genomes (*12*), and had been deposited in the GenBank database (Acc. No. MN326827). All elements in the ZC1310M genome were arranged in the order of 3'-Leader-N-P (V/C)-M-F-H-L-Trailer-5'. The BLASTn analysis revealed that the ZC1310M strain shared the highest (99.90%) and the lowest (92.71%) genomic identities with the isolate Hebei (GenBank: KC427278) and with the isolate Onderstepoort (GenBank: AF378705), respectively.

The ZC1310M H gene open reading frame (ORF, 1824 nt) was aligned with others available in the GenBank. These reference strains belonged to ten genetic lineages, including the Europe wild-life, Rockborn-like, Asia-4, Europe-1/South America-1, Asia-1, America-1 (Vaccines), Africa-1, Arctic, Asia-2 and Asia-3. Potential bias was avoided by selecting sequences across a wide geographic range. The aligned result was used to construct an H ORF-based phylogenetic tree using the Clustal W program of MEGA 7.0 software. The phylogenetic tree (Fig. 1a) revealed that the ZC1310M belonged to the Asia-1 lineage, within which it shared the highest nucleotide identity (99.7%) with the SD(09)3 (GenBank: HM448834), an isolate from Shandong Province, and the lowest nucleotide identity (99.1%) with the JL(07)4 (GenBank: EU564813), an isolate from Jilin Province. Out of all strains in Fig. 1a, the HLJ2 (GenBank: EU743935), isolated from a fox in Heilongjiang Province in 2005 (7), shared the lowest nucleotide identity (89.4%) with the ZC1310M. The ZC1310M H ORF was subsequently compared with others deposited in the GenBank using the BLASTn algorithm, consequently showing a 100% nucleotide identity with that of the isolate Hebei (GenBank: KC427278) from Hebei Province.

The ZC1310M was isolated from a dead mink, which had been inoculated with the live-attenuated vaccine (CDV3 vaccine strain) before appearance of its clinical signs. However, CDV3-specific sequences were not identified in the ZC1310M genome by next-generation sequencing. The alignment of their H ORFs showed that the ZC1310M shared a relatively low homology (91.4%) with the CDV3, implying that the ZC1310M was not derived from the CDV3 vaccine strain, but was a highly or moderately virulent isolate. The reason for immunization failure remained unclear.

Ten representative strains were independently selected from the ten genetic lineages in Fig. 1a for aligning deduced aa sequences (607 aa) of their H proteins by the Clustal W method using the MegAlign program of Lasergene 7.1 software. The alignment result (Fig. 1b) revealed that among these ten strains, the ZC1310M had seven unique aa substitutions: L25F, V47I, I157V, L171I, T245S, N277T and A359V. Especially, the ZC1310M showed one specific aa substitution, L25F, different from that of other strains in the Asia-1 lineage in Fig. 1a. A further BLASTp analysis revealed only one H ORF aa sequence (GenBank: AGI16936), which was fully identical to that of the ZC1310M.

N-linked glycosylation is a key factor in determining the antigenicity of some proteins, and usually occurs at asparagine residues in N-X-S/T sequons where X does not equal proline (*13*). In this study, potential N-linked glycosylation sites on the H protein were predicted by an online NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/#opennewwindow) for determining the difference among the ZC1310M and other isolates in Fig. 1b. The ZC1310M had nine such potential sites at aa positions 19–21, 149–151, 309–311, 391–393, 422–424, 456–458, 584–586, 587–589 and 603–605 (Fig. 1b), all of which were identical to those of Asia-1 lineage strains (*14*), and five (149–151, 422–424, 456–458, 587–589 and 603–605) of which were conserved among these ten strains in Fig. 1b.

Differences in N-linked glycosylation of the H protein may result in differences in neutralization efficacy, replication and virulence of CDV (*15-16*). The N-linked glycosylation site 309–311 is generally specific to field isolates but not to vaccine strains (*17*), and therefore is possibly relevant to CDV virulence. In this study, none of the America-1 (Vaccines) lineage strains in Fig. 1a had this glycosylation site, which was also absent in the Argentina 23 (GenBank: FJ392652), a field strain isolated from a naturally infected dog in Argentina.

Cysteine residues also play an important role in determining the H protein's structure, function and antigenic properties (*18*). Among all strains in Fig. 1b, cysteine residues were completely conserved at 12 positions (139, 154, 188, 283, 296, 377, 382, 390, 490, 566, 575, and 602) in the H protein. Actually, theses 12 positions were generally conserved among most CDV strains in the GenBank by BLASTp analysis. It has been reported that canid species are more likely to be infected by CDV strains with 549Y than with 549H in the H protein, whereas there is insufficient data available to test whether the probability of infection in non-canid species is significantly altered by the presence of either 549Y or 549H (*19*). The ZC1310M was isolated from a mink (non-canid host), and contained the 549Y in its H protein, identical to those of other strains in Fig. 1b.

In summary, the mink-origin CDV ZC1310M belonged to the Asia-1 lineage and displayed a high genetic homology with others in the same lineage. At the level of aa sequence, this isolate showed a unique aa substitution (L25F) on its H protein among all strains. Both N-linked glycosylation sites and cysteine residues were conserved in the H protein among the ZC1310M and other strains in the Asia-1 lineage.

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