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High Prevalence of Aleutian Mink Disease Virus in Free-ranging Mink on a Remote Danish Island

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ABSTRACT: Aleutian mink disease virus (AMDV) causes severe disease in farmed mink (*Neovison vison*) worldwide. In Denmark, AMDV in farmed mink has been confined to the northern part of the mainland since 2002. From 1998 to 2009, samples from 396 free-ranging mink were collected from mainland Denmark, and a low AMDV antibody prevalence (3% of 296) was found using countercurrent immune electrophoresis. However, on the island of Bornholm in the Baltic Sea, a high prevalence (45% of 142 mink) was detected in the free-ranging mink. Aleutian mink disease virus was detected by polymerase chain reaction in 32 of 49 antibody-positive free-ranging mink on Bornholm, but not in mink collected from other parts of Denmark. Sequence analysis of 370 base pairs of the nonstructural gene of the AMDV of 17 samples revealed two clusters with closest similarity to Swedish AMDV strains.

Key words: Aleutian mink disease virus, countercurrent immune electrophoresis, free-ranging mink, *Neovison vison*, PCR.

Aleutian mink disease virus (AMDV) causes severe disease in farmed mink (*Neovison vison*) characterized by acute fatal pneumonia in mink kits (Alexandersen, 1986), chronic immune complex–mediated disease (Porter et al., 1969), and impaired reproduction in adult mink (Hansen and Lund, 1988). The virus is widespread in farmed mink worldwide (Bloom et al., 1994; Olofsson et al., 1999). Infection has also been described in other domestic carnivores, including raccoons (*Procyon lotor*; Oie et al., 1996), striped skunk (*Mephitis mephitis*; Allender et al., 2008; Oie et al., 1996), and ferrets (*Mustela putorius furo*; Une et al., 2000).

Serologic evidence of AMDV has been found in free-ranging American mink, European mink (*Mustela lutreola*), polecats (*Mustela putorius*), stone martens (*Martes foina*), pine martens (*Martes martes*), and common genets (*Genetta genetta*; Cho and Greenfield, 1978; Yamaguchi and Macdonald, 2001; Fournier-Chambrillon et al., 2004). However, the virus has only been detected in free-ranging mink in Canada (Farid et al., 2010) and in Spain (Mañas et al., 2001). In Spain, AMDV was also detected in an otter (*Lutra lutra*), but histologic lesions were found only in free-ranging mink (Mañas et al., 2001). The nucleotide sequence of the capsid gene (VP2) of AMDV from these free-ranging mink was distinct from AMDV strains of ferrets and farmed mink (Mañas et al., 2001). We found a high prevalence of antibody to AMDV (45.1%) and demonstrated the virus in the population of free-ranging mink on the remote Danish island of Bornholm.

A total of 142 free-ranging mink from Bornholm (55°06′N, 14°54′E) and 396 free-ranging mink from the rest of Denmark were trapped or accidentally killed by traffic during 1998–2009. The mink were collected as part of the Danish Nature Agency's population control program for free-ranging mink, which are considered an invasive species to the Danish fauna. The mink ($n=538$) were necropsied according to standard procedures for mustelids. Blood was sampled from the heart in capillary tubes for serum separation. Sera from all mink were tested by countercurrent immune electrophoresis (CIE) by the Kopenhagen Diagnostics (Glostrup, Denmark) as described by Cho and Greenfield (1978). Spleen and mesenteric lymph nodes were sampled during

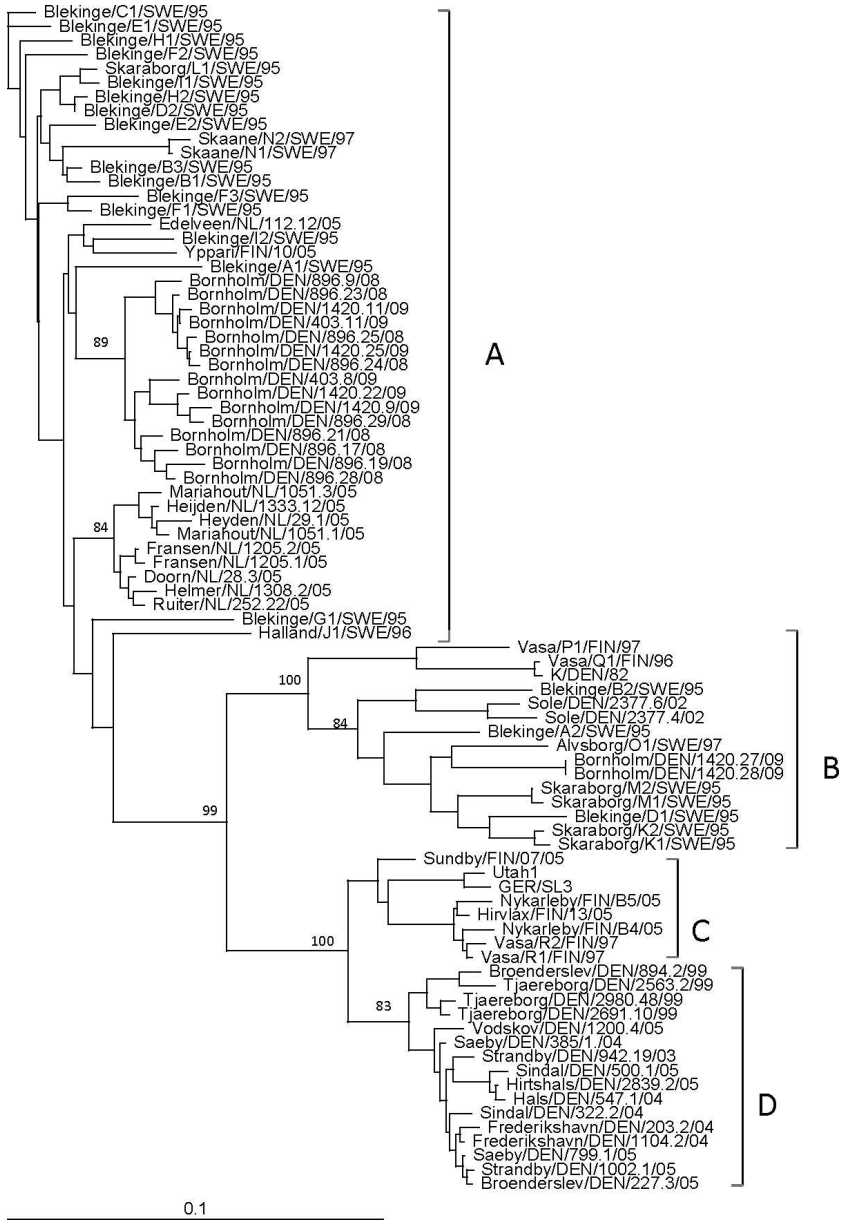

FIGURE 1. Phylogeny of the nonstructural gene (NS1) sequence of Aleutian mink disease virus (AMDV) from free-ranging mink (*Neovison vison*) from Bornholm, Denmark, showing clustering of the AMDV NS1 gene sequences into two groups. Groups as established by Olofsson et al. (1999).

2008–2009 from 57 mink and stored at −20 C until analysis by PCR.

The tissues were homogenized and treated with Qiagen protease (Qiagen, Hilden, Germany) as described by Jensen et al. (2011). DNA was isolated by QIAamp® Blood Mini kit according to manufacturer's instructions (Qiagen). The PCR was performed as described (Jensen et al., 2011) using the AMDV-F-7-HPN1 and AMDV-R-7-HPN2 primers amplifying a 370–base pair sequence of the nonstructural gene (NS1). Tissue from Dutch farmed mink infected with strain Edelveen/NL/

TABLE 1. Aleutian mink disease virus strains included in the nonstructural gene (NS1) phylogenetic analysis (see Fig. 1).

Isolate name, group	Accession No.	Reference
Bornholm, A and B	HQ435816-32	This study
Swedish and Vasa, A, B, C	AF107626-60	Olofsson et al., 1999
Finnish, A, C	EU908029-41	Knuuttila et al., 2009
Danish and Dutch, A, C, D	EU413693-3730	Christensen et al., 2011
K/DEN/82, B	X77084	Gottschalck et al., 1994
Utah1, C	X77083	Gottschalck et al., 1994
Ger/SL3, C	X97629	Schuierer et al., 1997

112.12/05 (GenBank EF413715.1) was used as a positive control. Tissues from Danish farmed mink from an AMDV-free area with no antibodies against AMDV were used as negative controls. Blank controls (water only and reagents only) were also included. To enable comparison of the sequences not only with published NS1 but also VP2 sequences, seven samples positive with the NS1 primers were tested with the primers described by Saifuddin and Fox (1996) and the conditions described by Jensen et al. (2011).

Sequencing was performed by LGC Genomics (Berlin, Germany) with the same primers as used for the PCR. The sequences were assembled and proofread using CLC DNA Workbench (http://www.clcbio.com/). Sequence similarity was detected by BLAST search (blast.ncbi.nlm.nih.gov). Sequence alignment was done using Clustal X (EMBL, Heidelberg, Germany; Thompson et al., 1997) using default parameters and 1,000 bootstrap trials. Aligned sequences were presented by GeneDoc (Nicholas et al., 1997), and dendrograms were visualized with TREEVIEW (Page, 1996) version 1.6.6. For simplicity, not all available sequences from GenBank were included in the NS1 phylogeny (Fig. 1), but relevant representatives of the groups established by Olofsson et al. (1999) and Christensen et al. (2011; Table 1) were included. Identical sequences were omitted in the figure with the exception of Bornholm/DEN/1420-27 and -28 because these sequences had minor differences in the VP2 gene sequences, although not in the NS1 gene sequences.

Mink on Bornholm had a significantly higher ($P<0.001$) prevalence of antibody to AMDV (64/142; 45.1%) than free-ranging mink from the rest of Denmark (13/396; 3.3%). The AMDV antibody prevalence in mink from Bornholm was similar on an annual basis from 1998 to 2009 (data not shown). We detected AMDV by PCR in 32 of 49 CIE-positive mink from Bornholm. No AMDV was detected by PCR in any antibody-positive free-ranging mink outside Bornholm ($n=8$). None of the mink showed any pathologic lesions indicative of AMDV infection.

The sequences of the AMDV strains from Bornholm clustered in two of the four groups: A and B (Fig. 1). The sequences in group A ($n=15$) had minimal variation between strains and formed a distinct group with a sequence difference of >5% from the closest relatives from Sweden within group A (Fig. 1; Olofsson et al., 1999). The two samples that clustered in group B had identical NS1 sequences and high similarity to the closest related Swedish AMDV strains. The AMDV sequences from Bornholm were different (>12% of NS1 sequence variation) from AMDV strains from Danish farmed mink (Christensen et al., 2011). Comparing the VP2 genes of the AMDV strains from Bornholm with existing VP2 genes, two distinct groups were found, but differences between sequences were

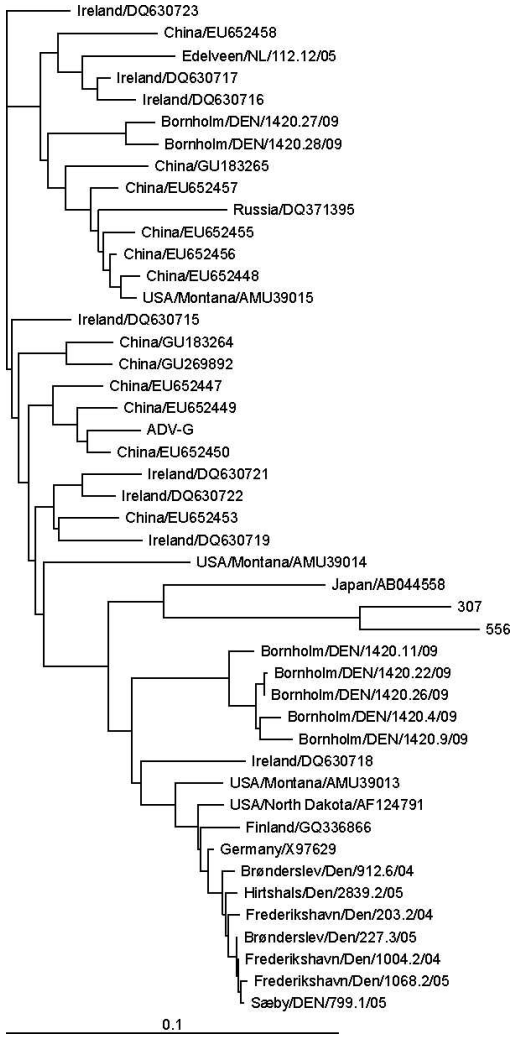

FIGURE 2. Phylogeny of the capsid gene (VP2) gene sequence of Aleutian mink disease virus (AMDV) from free-ranging mink (*Neovison vison*) from Bornholm, Denmark. Sequences cluster into two groups and are distinct from AMDV from Danish farmed mink. The sequences from the mink from Bornholm have GenBank accession numbers HQ435833-8. The other Danish sequences have not been submitted. The remaining sequences are all referred to with GenBank submission numbers.

generally larger than in NS1 sequences (Fig. 2). No Swedish AMDV VP2 sequences were available for comparison, but the NS1 Edelveen/NL/112 AMDV strain, which groups with Swedish AMDV strains of group A, was different when the VP2 sequences were compared (Fig. 2). GenBank accession numbers for the AMDV sequences in wild mink from Bornholm are: HQ435816–HQ435832 for the NS1 sequences ($n=17$) and HQ435833–HQ435838 ($n=6$) for the VP2 sequences. One VP2 sequence is not yet available in GenBank.

The two AMDV clusters from Bornholm and the similarities to Swedish AMDV strains indicate that there were two introductions of AMDV, most likely from transport of farmed mink from Sweden to Bornholm before the initiation of the Danish AMDV control program. The AMDV control program requires that all Danish farms are serologically screened for AMDV annually and no antibody-positive farmed mink have been detected on Bornholm for more than a decade. Free-ranging mink are considered to originate from escaped farmed mink; migration of free-ranging animals over a frozen Baltic Sea to Bornholm is highly unlikely. Therefore, the origin of AMDV in free-ranging mink most likely is farmed mink on the island at a time when AMDV was still circulating in the farmed mink.

The absence of pathologic lesions indicative of AMDV infection in the examined mink indicates that the AMDV strain Bornholm/DEN/08 is of low pathogenicity. This evidence is supported by the AMDV antibody prevalence of 45.1% and the fact that 82% of antibody-positive mink were also PCR-positive. This high prevalence of AMDV in free-ranging mink on Bornholm indicates that free-ranging mink can survive with circulating AMDV without showing classical clinical symptoms and mortality caused by Aleutian disease. Only antibody-positive mink from Bornholm were tested by PCR because earlier studies in farmed mink showed a high correlation between the CIE test and PCR, and the CIE is used as serologic screening (Jensen et al., 2011). Unfortunately, tissue was only available from one antibody-positive mink from the rest of Denmark because of severe lesions caused by traffic-related injury to the animals.

This antibody-positive mink was negative by PCR. This finding could be a result of decomposition of the animal or the animal could have cleared the infection and eventually it could be a false-negative result (Jensen et al. 2011). Another seven mink from Denmark (exclusively Bornholm) were tested by PCR and were all negative, even though the animals were chosen from the northern part of the country where AMDV is widespread in farmed mink.

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