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Mechanisms behind the varying severity of Aleutian mink disease virus: Comparison of three farms with a different disease status

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ABSTRACT

Aleutian mink disease virus (AMDV) is distributed widely among mink farms and wild mustelids despite ongoing attempts to stop the spread. The severity of Aleutian disease (AD) varies from subclinical to fatal but the reasons for its varying severity are complex and unclear. Recently, breeding of tolerant mink has drawn attention as the possible solution to reduce the effects of AD in farms. The aim of this study was to gather information on the effects of breeding based on overall health, production traits, and antibody titer on AD severity by comparing a positive farm (farm 1) that has been breeding for tolerance in mink to an infected farm without tolerance selection, and an AMDV-free farm. During the 2.5-year follow-up, the mink in farm 1 remained mostly free of clinical AD, had normal pelt quality and litter size, and had low virus copy numbers in tissues and low antibody titers in ELISA. In histopathological studies, most of the farm 1 mink had no/mild lesions in their kidneys. 29–43% of the mink were ELISA negative but PCR positive throughout the follow-up and frequent changes in virus strains and coinfections were observed. Several differences in gene expression between animals from different farms were also detected. These results indicate that the disease burden of AMDV can be reduced, with seemingly normal health and production rates, despite continual circulation of ADMV in cases where eradication attempts are unsuccessful.

1. Introduction

Aleutian mink disease virus (AMDV), species *Carnivore amdo-parvovirus 1* and family *Parvoviridae*, is widespread among farmed and feral mink (ICTVdb, 2021). AMDV has a 4.8 kb ssDNA genome that encodes five proteins. Left open reading frame (ORF) encodes structural proteins NS1, NS2, and NS3 that are needed for viral replication and right ORF encodes structural proteins VP1 and VP2 that form the capsid (Alexandersen et al., 1988; Bloom et al., 1990, 1988, 1980, 1982; Christensen et al., 1995; Huang et al., 2014). AMDV is transmitted horizontally directly or indirectly via body fluids like blood, feces, urine, and saliva or vertically through the placenta (Gorham et al., 1976; Jensen et al., 2014; Padgett et al., 1967). It is resistant to many standard physical and chemical treatments making it difficult to clear it from infected farms (Cho, 1976; Hahn et al., 1977; Hussain et al., 2014). AMDV has been found in a wide range of surfaces in infected farms,

including surfaces that are not in direct contact with infected animals (Prieto et al., 2017).

AMDV causes Aleutian disease (AD), an immune complex disease characterized by a massive number of antibodies and immune complexes that accumulate in tissues (Porter et al., 1969). Clinical signs and lesions in adult mink include anorexia, weight loss, reproductive failure, splenomegaly, lymphadenopathy, plasmacytosis, hypergammaglobulinemia, necrotizing arteritis, plasma cell-dominated mononuclear cell infiltrates in the organs, and glomerulonephritis, and vary in severity from transient to persistent and asymptomatic to fatal (Eklund et al., 1968; Porter, 1986). Antibodies are unable to neutralize the virus and no immunity from future infections is gained (Hadlow et al., 1984; Porter et al., 1969). There is no effective treatment and all attempts to develop a vaccine have been unsuccessful. Vaccines based on inactivated virus or capsid proteins and treatment with passive antibodies have only enhanced the disease, and vaccines based on NS1 led

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to partial protection, including lower death rates, that was insufficient to prevent the infection (Markarian and Abrahamyan, 2021).

Varying disease severity has been suggested to be connected to both viral and host factors. For example, the highly pathogenic Utah 1 strain usually causes a severe disease in mink homozygous recessive for Aleutian gene (Aleutian mink) as well as non-Aleutian mink whereas the Pullman strain causes severe disease mainly in Aleutian mink (Hadlow et al., 1983; Porter, 1986). Amino acid differences in capsid protein sequences between pathogenic strains and non-pathogenic AMDV-G have often been confined to a relatively small number of residues and a study by Kowalczyk et al. only detected one amino acid difference with the potential to affect the functionality of the protein in strains from two farms with subclinical and clinical infections (Bloom et al., 1988; Kowalczyk et al., 2018; Oie et al., 1996). It has been suggested that stabilization of the pathogen-host relationship plays a role in the clinical picture in farms as viral strains that eliminate the host are likely selected against as they do not provide for long-term transmission to be established in the population (Kowalczyk et al., 2018). When it comes to host factors, mink age and genotype both play a role in pathogenesis. In mink kits, disease manifests as acute interstitial pneumonia instead of the classic adult form of the disease (Alexandersen, 1986). Aleutian mink appear to be more susceptible to the disease than other genotypes and it has been estimated that ¼ of non-Aleutian mink can clear the virus (Eklund et al., 1968). Selecting mink based on phenotypic health and iodine agglutination test (IAT) has been shown to reduce the severity of lesions, and several farms in Canada have applied selection of disease tolerant mink as a form of disease control (Farid and Ferns, 2017; Farid and Hussain, 2020). Selecting mink that have low positive ELISA values has also been recognized (Andersson et al., 2017) and would be easily possible in Finland where ELISA is used for annual large-scale screening of AMDV in mink farms.

The aim of this project was to study the mechanisms behind varying clinical pictures by conducting a 2.5-year follow-up on asymptomatic mink from a farm that had been controlling the disease for decades through breeding AMDV tolerant herd. We studied viral loads, genetic

properties, transcriptomes from blood, pathological properties, and antibody response, and compared them to symptomatic mink from a farm without selection of tolerant mink and healthy mink from an AMDV-free farm.

2. Materials and methods

2.1. Farms and samples

Mink from three Finnish farms were sampled. Farm 1 had had AMDV since 1980 s and had been aiming to control the disease by selecting weak ELISA-positive but asymptomatic animals with normal litter size and pelt quality for breeding. Before ELISA, selection was done with iodine agglutination test (IAT). The farm reported having only an occasional mink with the clinical form of the disease and litter size was comparable to the average litter sizes in Finland. Farm 2 had had AMDV for a couple of years. They had recently had an acute rise in AMDV seroprevalence (18% half a year before sampling and 80% a month before sampling based on ELISA) and reported a few mink with AD per month and even more mink with signs possible caused by milder AD. Farm 3 was an AMDV-free farm based on annual ELISA testing.

Samples were collected according to Fig. 1. A long-term follow-up was performed in farm 1 by collecting rectal swabs and blood samples on filter paper by nail clipping in four occasions (May 2017, October 2017, October 2018, and November 2019). Follow-up started with 10 white, brown, and sapphire mink but ended with six brown, five sapphire, and six white mink since 13 mink for unreported causes died during the follow-up. Five white mink showing signs of anorexia and dehydration were selected from farm 2 and seven healthy white mink from farm 3. For the euthanasia (final sampling in farm 1 and samplings in farms 2 and 3), mink were sedated with intramuscular dosing of 0.4 ml of 10 µg/ml medetomidine (Domitor, Orion Pharma, Espoo, Finland) combined to 0.4 ml of 50 mg/ml ketamine (Ketalar, Pfizer animal health, Helsinki, Finland) after which serum (BD Vacutainer, Plymouth, UK) and blood samples (Tempus blood RNA tubes, Applied Biosystems, Foster city, CA,

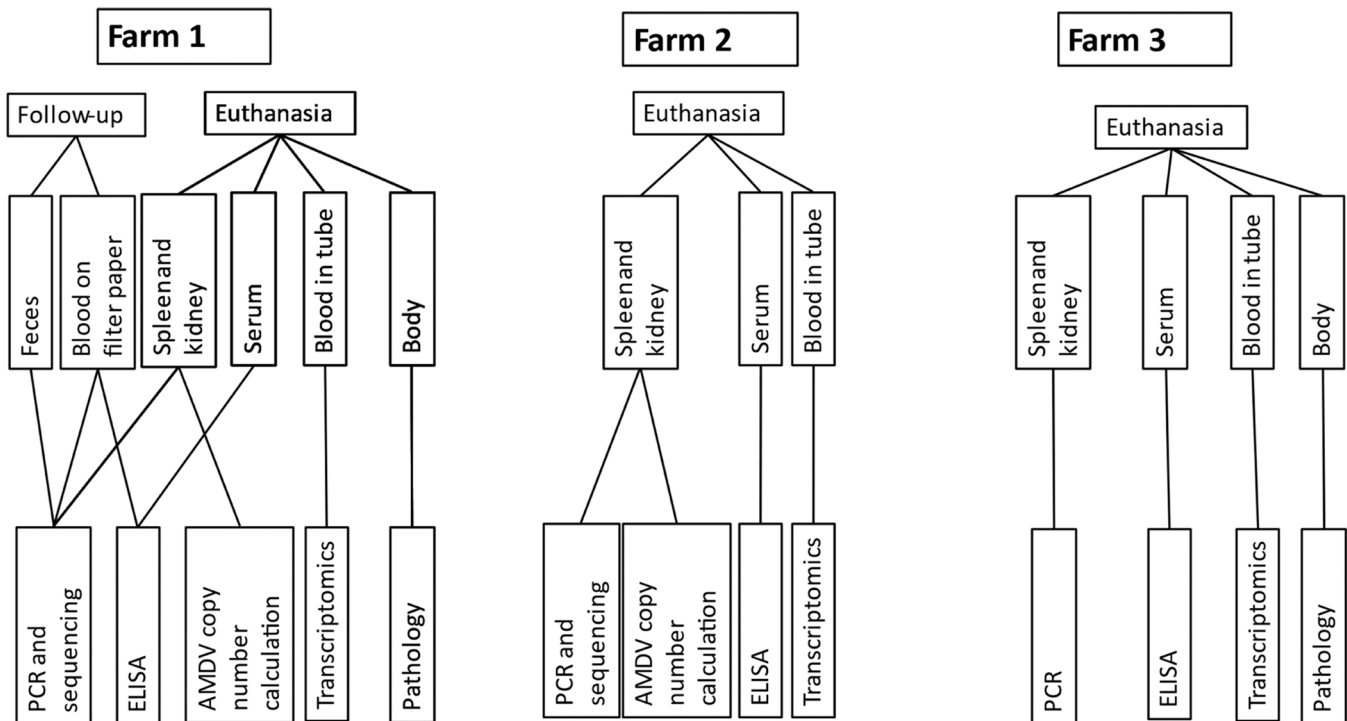


Fig. 1. Summary of the samples and methodology. Mink from farm 1 (with decades of selecting AMDV tolerant mink) were sampled in four occasions between March 2017 and November 2019 (follow-up). Euthanasia samples were taken from farm 1 and control farms 2 (no tolerance selection) and 3 (AMDV negative farm) in late 2019.

USA) were taken by cardiac puncture. Mink were euthanized with 2 ml of intracardial pentobarbital sodium (Euthasol, 400 mg/ml, Le Vet B.V., Oudewater, the Netherlands). The health of the mink before samplings were assessed, samples taken, and the necropsies performed by a veterinarian.

2.2. Pathological examination

Mink (n = 17) from farm 1 and two of the control mink from farm 3 were submitted for necropsy for gross and histopathological examinations. Body weight and weight of the spleen of each mink were measured. Spleen, kidneys, liver, lungs, heart, stomach, small intestine, colon, thyroid glands, adrenals, bladder, muscle, mesenteric lymph nodes, brain, hypophysis, bone marrow, and all other tissues with possible abnormalities based on macroscopic evaluation were sampled for histopathological studies. The samples were fixed in 10% neutral buffered formalin, processed routinely, and stained with haematoxylin-eosin. Spleen and kidneys were also sampled for PCR. Detected gross and histopathological changes in organs were scored from 0 to 3 (0 = no lesions, 1 = mild lesions, 2 = moderate lesions, and 3 = severe lesions).

2.3. DNA extraction

DNA was extracted from spleen and kidney tissues with NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany) with a standard protocol for tissue samples and from blood samples by incubating approximately 1 cm² piece of filter paper in 300 µl of PBS o/n at 4 °C and using support protocol for viral DNA from blood samples for DNA extraction. DNA was extracted from stool samples with QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) or with QIAcube HT using DNeasy 96 PowerSoil Pro QIAcube HT Kit (Qiagen, Hilden, Germany).

2.4. PCR, quantitating, and sequencing of virus strains

Follow-up samples (blood on filter paper and feces) from farm 1 were tested with SYBR green-based pan-AMDV-PCR, which amplifies partial NS1 (nt 578-951) (Jensen et al., 2011; Virtanen et al., 2019) and pan-AMDO-PCR, which amplifies partial NS1 and NS2 (nt 1662-2302) (Knuutila, 2015) (sites according to AMDV-G (M20036.1) throughout the manuscript). Those samples that were positive with at least one PCR were considered positive. Spleen and kidney samples were screened with pan-AMDV-PCR. Pan-AMDV-PCR products from all positive spleen and blood samples, and kidney samples from mink that were negative from spleen were sequenced with Sanger sequencing. All the real-time PCRs were performed with Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA). PCR products were purified for sequencing by adding 0.5 µl of Exonuclease I and 1 µl of FastAP Thermo-sensitive Alkaline Phosphatase (Thermo Scientific, Vilnius, Lithuania) to 5 µl of PCR reaction and incubating them at 37 °C for 45 min and 85 °C for 15 min.

To determine AMDV copy numbers, spleen and kidney samples were tested with quantitative NS1-probe-PCR (Virtanen et al., 2020). Each run contained a dilution series (10⁴, 10³, 100, 10, 1, and 0 copies/reaction) of a plasmid containing the PCR product prepared earlier (Virtanen et al., 2020) in three parallel reactions and the samples in two parallel reactions. DNA concentrations were measured with NanoDrop. If both parallel reactions were positive, their average Ct-value with a 0.04 cut off was used in calculations. If one reaction was positive and the other one was negative, the negative reaction was excluded.

2.5. Serology

All the blood on filter paper samples and serum samples were tested with AMDV VP2 ELISA (Knuutila et al., 2009). Blood was extracted from filter paper by incubating a circular piece in 200 µl of dilution buffer (PBS + 0.5% BSA + 0.05% Tween 20) o/n, and undiluted liquid

was used for ELISA. Sera were diluted 1:200. Peroxidase-conjugated AffiniPure Goat Anti-cat IgG (H + L) (Jackson ImmunoResearch, West Grove, PA, USA) with 1:4500 dilution or Goat anti-ferret IgG (H + L) secondary antibody (Novus, Abingdon, UK) with 1:20 000 dilution was used as a conjugate. ELISA cut-off was determined separately for each conjugate with a panel of 10 negative samples in seven replicates and adding two standard deviations to the mean absorbance. Each ELISA analysis included at least two blank wells (with dilution buffer only), the mean value of which was subtracted from the sample absorbances before the analysis.

2.6. Testing for mink circovirus

Due to indications that circoviruses might increase the severity of secondary infections (Todd, 2000), the mink were tested for mink circovirus (MiCV). PCR reactions contained 12.5 µl of Fermentas SYBR MasterMix (Thermo Scientific, Vilnius, Lithuania), 6 µl of H₂O, 0.75 µl of 10 µM primers CapF and CapL (Cui et al., 2018) and 5 µl of DNA isolated from spleen. The mixture was incubated at 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 1 min at 55 °C and 1 min at 72 °C, and melting curve analysis with 1 min at 95 °C, 30 s at 55 °C and 30 s at 95 °C. PCR products with unclear results were purified as earlier and sequenced with Sanger sequencing as described above.

2.7. Transcriptome analysis

Three mink from each farm (F1/27, F1/29, F1/30, F2/1, F2/2, F2/4, F3/3, F3/4, and F3/6) were subjected to transcriptome analysis. All nine mink were white females. RNA was isolated from the blood samples (Tempus blood RNA tubes) with Tempus Spin RNA Isolation Kit (Thermo Scientific, Vilnius, Lithuania) and its quality checked with Bioanalyzer (Agilent, Santa Clara, CA, USA). Libraries were prepared with TruSeq Stranded Total RNA kit (Illumina, Eindhoven, the Netherlands) and ribosomal RNA were depleted using human Ribopools (siTOOLsBiotech, Planegg, Germany). Single end reads were generated from the libraries obtained on NextSeq using a 75 bp kit v 2.5 (Illumina, Eindhoven, the Netherlands). The libraries and sequencing were performed at the DNA Sequencing and Genomics Laboratory, Institute of Biotechnology, University of Helsinki. Reads were then trimmed using Trimmomatic (Bolger et al., 2014) followed by quality check with FASTQC (Andrews, 2010). Then reads were aligned to the mink (*Neogale vison* GCF.020171115.1) reference genome using Bowtie2 (Langmead and Salzberg, 2012), followed by counting aligned reads per gene using HTSeq (Anders et al., 2015). Finally, we checked for differentially expressed genes (DEG) using edgeR (Robinson et al., 2010) (P-value cutoff 0.05). All the analysis was done in R-4.1.1 (R core team, 2021).

2.8. Data analyses

Statistical analysis was performed with IBM SPSS statistics 27. Normality of the data was assessed with Shapiro-Wilk test and equality of the variances with Levene's test (significance level of 0.05). Differences of means between independent groups of normally distributed data were tested with Independent-Samples T-test (two groups) or one-way ANOVA (more than two groups). Pairwise differences of one-way ANOVA were determined with Bonferroni analysis. If the data was not normally distributed, corresponding analysis was performed with Mann-Whitney U-test (two groups) or Kruskal-Wallis test (more than two groups). Correlations between variables were assessed with Spearman's rho values.

Poor quality Sanger sequences and sequences that seemed to have two or more overlapping sequences were excluded from the sequence data and the rest of the AMDV sequences were submitted to GenBank under accession numbers OM142153-OM142203. Sequences were aligned with MEGA6 (Tamura et al., 2013) using ClustalW (Thompson

et al., 1994) together with Finnish strains published in GenBank and a representative of strains from other countries (picked based on a tree built with all published AMDV-sequences of nt 578–951 (Virtanen et al., 2019). Phylogenetic tree without molecular clock was built with BEAST 1.8.2 (Drummond et al., 2012), Tracer v1.6 (Rambaut et al., 2014), and FigTree v1.4.2 (Rambaut, 2014) with a 20,000,000 chain length, Hasegawa-Kishino-Yano model (HKY+G, picked as the best fitting evolutionary model by MEGA6), and Bayesian skyline. To analyze the molecular clock, an additional tree, containing all Finnish sequences with known sampling years and months, was built using same parameters and a lognormal relaxed clock. Effective sample size values were checked to be over 100.

3. Results

3.1. IgG levels were consistently below the detection limit in several asymptomatic PCR positive mink

The proportion of antibody positive mink in farm 1 varied between 44% and 71% and PCR positive mink between 52% and 100% during the follow-up period (Table 1, detailed information about individual samples in Table S1). 30% (8/27) of mink from farm 1 that were ELISA negative were PCR positive in March 2017, 29% (8/28) in October 2017, 43% (9/21) in October 2018, and 41% (7/17) in November 2019 (Fig. S1). Only up to 30% of tested feces samples were PCR positive. All mink from farm 2 were positive and farm 3 were negative both in ELISA from serum and PCR from spleen and kidney. 13/17 mink from farm 1, all mink from farm 2, and one out of two tested mink from farm 3 were MiCV positive. Due to almost all the mink being positive, correlations between MiCV and AD severity were not further analyzed. No correlations between absorbances in ELISA and the litter sizes of same years were detected (Fig. S2).

3.2. Mild histopathological lesions were detected in asymptomatic, AMDV-positive mink

In the necropsy, 14 mink from farm 1 were classified as fat, two as in normal body condition, and one (F1/D) as thin. Both control animals from farm 3 classified as fat. Spleen weight as a percentage of body weight was higher in sapphire (mean=0.81%, SD=0.21, n = 5) mink as compared to brown (mean=0.29%, SD=0.11, n = 6, and p = 0.026) and white mink (mean=0.24%, SD=0.086, n = 6, and p = 0.033) (Fig. S3). In farm 3, values were similar to those of white mink from farm 1 (mean=0.21%, SD=0.038, n = 2). In gross pathological examination, sapphire mink F1/D had findings typical for AD i.e., cachexia, dehydration, pale, enlarged and mottled kidneys, and splenomegaly (Fig. S4). No significant macroscopic lesions typical of AD were detected in other mink from farm 1 or AMDV negative control mink from farm 3, but mild unspecific changes were observed in some of the mink (fatty liver, hyperemia in gastrointestinal track, occasional enlarged lymph nodes etc.).

In histopathological examination, both AMDV negative control mink had mild or moderate lesions in lungs (peribronchial and perivascular infiltration of mononuclear cells) and spleen (extramedullary hematopoiesis) (means=1.50). One of them had mild chronic eosinophilic enteritis in the intestines and one mild lipidosis in the liver (mean=0.5).

Table 1

Percentage of positive samples in PCR and ELISA in farm 1 during follow up. Number of positive individuals/number of tested individuals is reported in parentheses.

	ELISA	PCR (blood)	PCR (feces)
March 2017	40.7 (11/27)	51.9 (14/27)	30.0 (3/10)
October 2017	71.4 (20/28)	100.0 (28/28)	18.2 (2/11)
October 2018	52.3 (11/21)	95.2 (20/21)	0.0 (0/8)
November 2019	58.8 (10/17)	100.0 (17/17)	5.9 (1/17)

Neither of the mink had lesions in kidneys, brain and meninges, or in other organs. In farm 1, lesions were most severe in the spleen, followed by liver and lungs. Lesions were also detected in kidney and brain (Table 2, Figs. S5 and S6, and Table S3). The clinically sick mink (F1/D) had typical lesions for Aleutian disease, i.e., severe arteritis in various organs, severe glomerulonephritis, and moderately increased number of plasma cells in the spleen. Accumulations of mononuclear cells, predominantly plasma cells on heart, liver, kidneys, and gastrointestinal tract, as well as perivascular cuffing in the meninges, were also detected. Lesions in clinically healthy mink were generally milder or absent (Fig. 2). Mild to moderate non-specific changes were observed in the kidneys (calcification, chronic interstitial nephritis), spleen (extramedullary hematopoiesis, congestion), liver (chronic (neutrophilic) cholangiohepatitis, lipidosis), lungs (congestion, alveolar edema), and intestines (chronic eosinophilic enteritis). No changes were observed in skeletal muscle, thyroid, or adrenal glands in any of the mink and they were not included in out analysis.

3.3. AMDV genome copy number in tissues was higher in a farm with clinical disease

In total, 12/17 spleen samples and 15/17 kidney samples of mink from farm 1 were PCR positive. AMDV genome copy numbers (as copies/ng of DNA) were quantitated from 13 kidney and 8 spleen samples whereas quantitation of the rest of the positive samples was unsuccessful due to too small amount of DNA (Table 3). All five spleen and kidney samples from farm 2 were PCR positive and successfully quantified. Copy number was significantly higher in farm 2 as compared to farm 1 in both tissues (Fig. 3).

3.4. IgG levels correlated with virus copy number in spleen and spleen weight

IgG levels, virus copy numbers, spleen weight, and histopathological lesions were compared to find possible correlations (Table 4 and Figs. S7–S9). Spleen weight (as proportion of body weight) had a positive correlation with IgG levels in serum. IgG levels also showed positive correlation with virus copy number in the spleen and possible but not statistically significant correlation with copy number in the kidney.

3.5. Sequencing results

2/27 blood samples from farm 1 showed signs of two or more overlapping sequences in March 2017, 15/28 in October 2017, 12/21 in October 2018, and 7/17 in December 2019. In total, sequences from this

Table 2

Summary of histopathological studies of mink from farm 1.

Tissue	N ^a	Mean b	Range	Changes
Spleen	13/ 14	1.43	0–2	Extramedullary hematopoiesis, infiltration of plasma cells in the red pulpa
Kidney	10/ 17	0.71	0–3	Glomerulonephritis, mild focal or multifocal infiltrates of mononuclear cells
Liver	14/ 17	1.00	0–3	Focal or multifocal infiltration of mononuclear cells on portal tracts
Lungs	11/ 13	1.00	0–2	Peribronchial and perivascular infiltration of mononuclear cells
Intestines	12/ 17	0.71	0–1	Infiltration of mononuclear cells and perivascularitis in the lamina propria
Brain	1/ 17	0.11	0–2	Multifocal perivascular cuffing of mononuclear cells

Results of individual mink can be viewed in Table S1 and results sorted by color type in Table S3.

^a Number of mink with lesions/number of mink with a readable sample.

^b Mean severity of lesions (0 =no lesions, 1 =mild lesions, 2 =moderate lesions, and 3 =severe lesion).

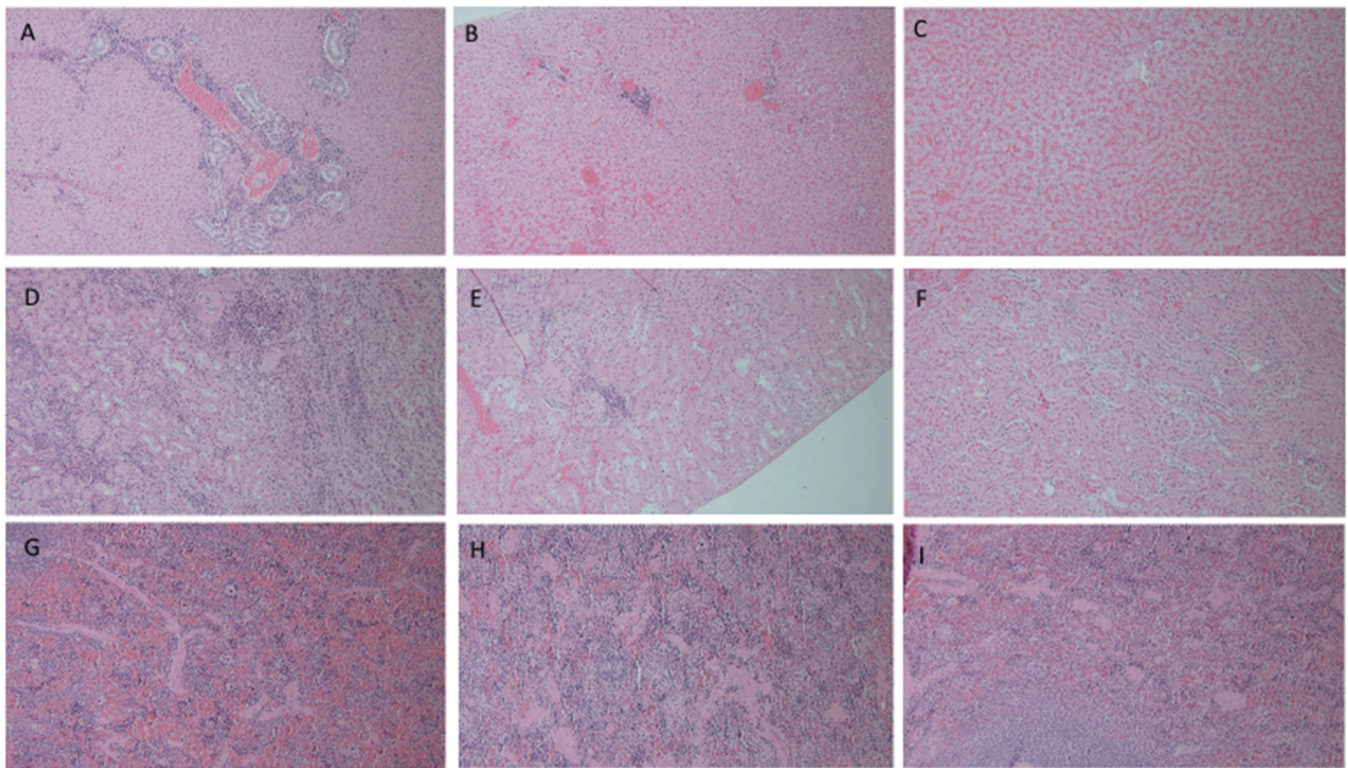


Fig. 2. Histology of a mink with clinical signs of AD (A, D, and G) and a clinically healthy AMDV-positive mink (B, E, and H) from an infected farm (farm 1), and a non-infected mink from AMDV-negative farm (C, F, and I; farm 3): moderate to severe multifocal perivascular and periportal plasma cell-rich mononuclear cell infiltration and biliary duct hyperplasia in liver (A), mild multifocal infiltration of mononuclear cells around blood vessel in liver (B), normal liver parenchyma (C), moderate multifocal lymphoplasmacytic interstitial nephritis in kidney (D), mild multifocal periglomerular aggregate of mononuclear cells in kidney (E), normal kidney (F), moderate extramedullary hematopoiesis in spleen (G), mild extramedullary hematopoiesis in spleen (H), and normal spleen (I) (all pictures 100x H&E).

Table 3

Average copy numbers per ng of DNA in samples of PCR-positive mink from farms 1 and 2. Samples with copy numbers of 0 have not been included.

Farm	Color	Tissue	Average	SD	N
Farm 1	Combined	Spleen	948.66	3283.45	10
		Kidney	1204.7	4337.78	15
	Brown	Spleen	1.82	2.87	3
		Kidney	1.33	0.8	5
	Sapphire	Spleen	3791.97	6567.09	3
		Kidney	5218.1	7818.45	4
	White	Spleen	474.6	0.73	4
		Kidney	0.07	0.06	6
Farm 2	Sapphire without F1/Da	Spleen	0.46	0.61	2
		Kidney	4.82	6.74	3
	White	Spleen	545.87	681.91	5
		Kidney	3302.22	7369.26	5

^a Due to one of the sapphire mink (F1/D) having severe clinical AD, copy numbers are also shown by only counting clinically healthy individuals.

study locate in five different branches in phylogenetic tree (Fig. 4, Fig. S11) with strains from farm 1 locating in branches I-IV and strains from farm 2 in branch V. Virus strains changed in at least nine of the mink during the follow-up based on the sequencing from blood. From the 2019 sampling, usable sequences from both blood and tissue (spleen or kidney) were acquired from seven mink. In three of those, the sequences in spleen/kidney and blood were similar whereas in others, 2–13 nt differences were observed. The most recent common ancestor (tMRCA, Fig. S10) was 21.2 years (95% HDP=9.08–35.34) for strains from farm 1 and 4.71 years (95% HDP=3.19–7.15) for strains from farm 2. tMRCAs for clusters I-IV were 7.13 years (95% HDP=2.97–14.01 years), 10.41 years (95% HDP=3.33–20.41 years), 11.62 years (95% HDP=3.66–22.21 years), and 2.96 years (95% HDP=2.23–3.96 years).

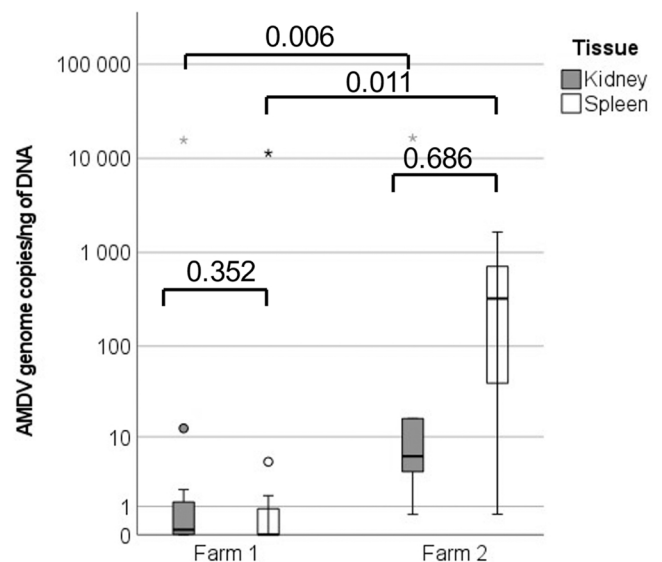


Fig. 3. Comparison of AMDV genome copy numbers in kidney and spleen samples between farms 1 (N = 17) and 2 (N = 5) and between tissues.

3.6. Transcriptomes

Transcriptome analysis was used to study differences in gene expression between the farm selecting tolerant mink (farm 1) and the farm without selection (farm 2) (Table 5, Table S4). As compared to an AMDV free farm (farm 3), less differentially expressed genes (DEGs) were detected in farm 1 than in farm 2. Most of the DEGs were unique

Table 4

Spearman correlation coefficients between ELISA, spleen weight, and quantitation results. P-values are shown in parenthesis and significant values are bolded.

	Copy number in spleen	Copy number in kidney	Copy number in blood	A450 (Serum)	Spleen weight (%)
Copy number in spleen	1	0.111 (0.671)	0.040 (0.880)	0.568 (0.017)	0.078 (0.765)
Copy number in kidney	0.111 (0.671)	1	0.086 (0.742)	0.375 (0.138)	0.384 (0.128)
Copy number in blood	0.040 (0.880)	0.086 (0.742)	1	0.311 (0.224)	0.249 (0.335)
A450 (Serum)	0.568 (0.017)	0.374 (0.138)	0.311 (0.224)	1	0.589 (0.013)
Spleen weight (%)	0.078 (0.765)	0.384 (0.128)	0.249 (0.335)	0.589 (0.013)	1

(only detected in one farm) and only seven genes (DDIT4, DTHD1, GZMA, LOC122900174, LOC122915485, STYK1, and WSCD2) were upregulated and none were downregulated in both infected farms. HPGD and NNMT were upregulated in farm 1 but downregulated in farm 2. The genes that had the highest differential expression ($\log_2FC > 4$ or < -4) had varying functions in immune response, apoptosis, differentiation, transcription regulation, signaling, development, and other processes (Table 5).

A closer look was taken at the DEGs connected to immune response. In addition to the few highly differentially expressed genes listed in Table 5, several other immune response genes were also upregulated in mink from farm 2. These include a proinflammatory cytokine IL-1 β and IL-27 that has several pro- and anti-inflammatory properties like inducing Th1 response and suppressing Th2 and Th17 responses. Receptors for IL-12 (stimulating NK cells and inducing differentiation of Th1 cells), IL-2 and IL-10 (negative regulation on inflammatory response and positive regulation of T- cell development), and IL-15 (differentiation of natural killer cells) were also upregulated as well as IRF7 (activation of interferons α and β), CXCL8, CXCR6, and CCL5 (chemotaxis), CD8A (T cell activation), and TNFAIP3 (a negative regulator of innate immune response). Genes that were downregulated in farm 2 included IL1RL1 (negative regulation of Th1 response and positive regulation of inflammatory response), TNFRSF13C (positive regulation of B and T cell proliferation), and IL5RA (B cell proliferation). Most of these DEGs were not detected in farm 1.

4. Discussion

In this study, we report a comparison of mink from three farms, one of which had been living with AMDV for decades and had mostly asymptomatic mink, one that had been infected for a couple of years and had mink with clinical AD, and one that was AMDV-free. The results show correlations and differences between antibody response, viremia, virus copy numbers, pathology, and transcriptomes between the tree farms. We also conducted a 2.5-year follow-up in farm conditions in the farm with a long history of breeding tolerant mink and observed a high proportion of mink that remained ELISA negative but PCR positive throughout the follow-up.

Due to the lack of vaccine or treatment, AMDV control relies greatly on diagnostics with serological tests like CIEP and ELISA. Interestingly, up to 43% of ELISA negative mink were PCR positive from blood in our follow up. A high proportion of antibody-negative and PCR positive mink has also been noted earlier by Farid et al. who found that 16.5% and 40.0% of CIEP negative mink were PCR positive from spleen (Farid, 2020; Farid and Ferns, 2017). In China, 12.5% of CIEP negative mink from 5 farms were PCR positive from spleen (Wang et al., 2014) and in Sweden, 4.5% of free-ranging mink were ELISA negative but PCR positive from spleen (Persson et al., 2015). As the sensitivity of ELISA has been shown to be comparable to that of CIEP (Andersson and Wallgren, 2013; Knuutila et al., 2009), differences in the protocol are unlikely to fully explain the high proportion in our study. One possible explanation for the ELISA negative results of AMDV-infected individuals would be fresh infections, as detectable antibody response may form later than detectable viremia (Farid et al., 2015). However, it is most likely not the only explanation, as many of the mink remained ELISA negative throughout the follow-up. It is also likely that decades of breeding have

led to the selection of low antibody producers, as was also suggested by Farid et al. (Farid and Ferns, 2017). It should be noted that ELISA negative results does not necessarily mean no AMDV antibodies at all, but it means that they were below the detection limit that was determined using a commonly accepted guideline. It has also been shown that in some cases (e.g. low inoculum doses), antibody titer may decrease over time (Farid and Hussain, 2020), but as these mink are constantly re-exposed by diverse strains circulating in the farms, that is not the most likely explanation for the low antibody titers in this study. Mink from farm 1 showed some fluctuation in antibody titers and also occasional high titers despite being asymptomatic, which is not unheard of, as asymptomatic non-Aleutian mink may also have at least transiently high antibody titers (Bloom et al., 1975).

Due to decades of breeding, it is difficult to say how common consistently seronegative but PCR positive mink are in other farms, but false negative ELISA/CIEP results would explain the unsuccessful attempts to fully eradicate AMDV from infected farms. Further studies should be conducted on ELISA/CIEP negative mink in other AMDV infected farms with different disease status (freshly infected naïve animal population vs long-term infected farm with established pathogen-host relationship) to determine the extent of this phenomenon, as false negative results in diagnostics greatly hinder the eradication attempts. These results and the results from previous studies (Farid and Ferns, 2017) also bring up the option to co-exist with the virus by breeding tolerant mink as eradication has proven to be difficult and no fully effective vaccine exists. However, virus may cause problems when entering the naïve non-tolerant mink population, which is supported by the fact that farm 1 reported usually temporarily having more symptomatic mink when new mink were introduced into the farm (personal communication).

Previous studies have shown that AMDV can persist in tissues even when viremia in blood, feces, and mouth is transient (Farid and Hussain, 2020; Jensen et al., 2014). After the first sampling, we consistently detected virus in blood samples, which is somewhat contradictory to that finding, but may be explained by the constant re-exposure to the virus in this study as compared to experimental infections. AMDV was, on the other hand, only transiently detected in feces which indicated that mink did not frequently shed the virus in feces. Phylogenetic analysis from blood showed the virus strains frequently changed between samplings. Different sequences in different samplings can result either from virus evolution within the host (Canuti et al., 2016; Virtanen et al., 2019) or from clearance of one virus strain and infection with another. In this case, the reason is probably a combination of both. In many cases, virus strains locate in totally different branches, with tMRCA being up to 21 years in different samplings, which speaks more for virus clearance and reinfection by another strain. Sanger sequence raw data also frequently showed overlapping AMDV sequences, most likely resulting either from coinfection or within-host evolution. Another interesting observation was different virus strains in tissues and blood, but this result is not uncalled for as the same phenomenon has been detected with other viruses like HIV (Haggerty and Stevenson, 1991) and can result from either coinfection or within-host evolution. As the farm has been infected for an exceptionally long time, great variation in virus strains is not a surprise, considering AMDV has been shown to have an exceptionally high substitution rate, most likely due to the intense farming practices (Virtanen et al., 2019). Multiple introductions

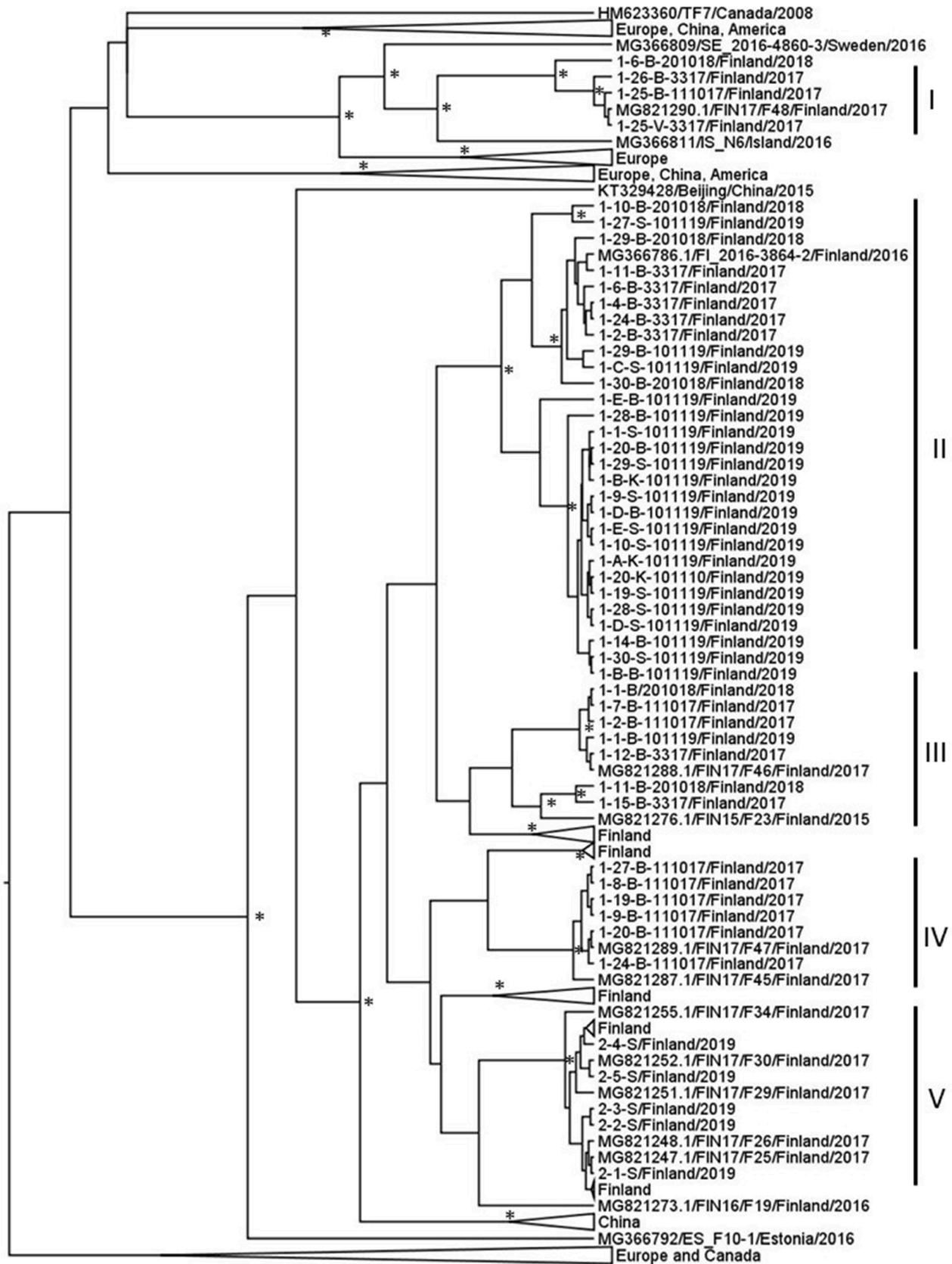


Fig. 4. Phylogenetic tree from nt 578–951 containing sequences from this study, previously published Finnish strains, and selected strains from other countries from GenBank. Sequences from this study have been named as follows: farm id (1 or 2) - mink id (1–30 and A-F) - sample material (B=blood, K=kidney, and S=spleen) - sampling date. Branches with posterior values above 0.9 have been marked with * and strains from this study have been divided into clusters I-V. Branches that don't contain sequences from this study have been collapsed for the sake of clarity and the full tree can be viewed in [supplementary material](#).

Table 5

Numbers of up- and downregulated genes detected between farms with different AMDV statuses. Results are expressed as numbers of genes with p-values below 0.05 in differential expression analysis was performed with edgeR. Highly up/downregulated genes ($\text{Log}_2\text{FC} < -4$ or >4) are listed separately and the farm against which the comparison is made is reported in parenthesis.

	Upregulated genes			Downregulated genes			
	N	High difference genes	Function	N	High difference genes	Function	
Farm 1 (farm 3)	63	LMCD1	Transcription	136	COL22A1	Structural protein	
		PRUNE2	Apoptosis				
Farm 2 (farm 3)	237	CD160, OSM, DDIT4, GZMA	Immune response	184	TUB	Phagocytosis	
		ARNT2, BATF2, COL19A1, GATA6, GLI2, NAMPT, ONECUT2	Differentiation, cell cycle, and transcription		FBXL2, LEXM	Other immune response functions	
		LRRRC7	Adhesion		ADCYAP1R1, MAPK4	Differentiation, cell cycle, and transcription	
		SCN5A	Ion transport		EDIL3	Adhesion	
		NUGGC	Nucleotide binding		NXF5	RNA transport	
		CAPN14	Proteolysis		APCD1, SORCS1	Signaling	
		GOS2	Apoptosis		TFPI2	Blood coagulation	
		ELMOD1, GALNT13, LGSN, WSCD2, CCDC178	Other/unclear function		ACP7, CFAP43, LYPD1	Other/unclear function	
		NR4A2, ONECUT2, POU2F3	Transcription		237	ADCYAP1R1	Differentiation
		CSTA	Adhesion		CDH5, EDIL3, PCDH7	Ahesion	
Farm 2 (farm 1)	294	CD177, LCN2, PGLYRP1, S100A12	Innate immunity	FBXL2	Virus-host-interaction		
		GOS2, NOL3	Apoptosis	COL28A1, LYPD1, NXPH1	Signaling		
		OSM	Liver development	FCER1A	IgE-binding		
		SCN5A	Ion transport	MAPK4	Cell cycle		
		COL4A1, COL22A1	Structural protein	TUB	Phagocytosis		
		PLAUR, DCLK1, NUGGC, CATSPERB, SCN5A, CCDC178, LGSN, LRRRC18, NAMPT, MYO16, GALNT13, ELMOD1, FOS	Other/unclear function	PALD1, OCLN, AASS, HS6ST2, HPGD, SORBS2	Other/unclear function		

into the farm are another possibility.

After years of selecting low antibody producers for breeding, farm 1 appeared to have been able to breed an AMDV-tolerant herd and their mink had litter sizes and pelt quality comparable to the average in Finland. Mink often produced low number of antibodies and had lower copies of virus in their tissues as compared to the mink from farm 2. In histopathological evaluation, mink from farm 1 had mild lesions similar to the lesions seen in AMDV infections in kidneys, spleen and liver, indicating that virus or immune complexes may have caused some tissue damage despite the lack of visible symptoms. Also, one of the mink that remained healthy most of the follow-up developed severe AD with typical histopathological lesions by the last sampling despite the fact that the farmers reported rarely having mink with clinical AD. The virus strain of that mink was the same as in some of the asymptomatic mink so the change of virus strain to a more pathogenic one is not the most likely explanation for the sudden onset of symptoms. More likely, this might be connected to the fact that mink were kept alive longer than they normally would have been. Possibly the tolerant mink were not completely unaffected by the virus but had a very slowly progressing form of the disease and some of the other mink in the follow up may have also developed AD if the follow-up had been continued. Kidneys have a good reserve capacity, and clinical signs of kidney failure may not be detected until kidney function declines to 25% or less (Cianciolo and Mohr, 2015).

With regard to differences between color types, some of the previous studies have detected more antibodies in Aleutian type mink (Porter et al., 1984). We did not detect higher ELISA absorbances in sapphire mink and, on the contrary, white mink had the highest mean ELISA values in ¼ samplings (Fig. S1). Breeding might play a role in low numbers of antibodies, even in sapphire mink. Also, we did not observe

differences in AMDV genome copy numbers in asymptomatic, AMDV-positive mink of different color types (Table S2). However, our results are influenced by the fact that we focused on mainly asymptomatic mink (excluding one sick mink in the last sampling) and the results might be different in a naïve mink population that has not been previously exposed to the virus. A proper comparison of color types would also require a larger sample size. The only difference detected between color types was that sapphire mink, which are considered more susceptible to the disease, had larger spleens in relation to their body weight. This is logical considering that AMDV infection is known to cause enlarged spleen both in farmed and free-ranging mink (Eklund et al., 1968; Zalewski et al., 2021), but a better comparison would require the inclusion of uninfected controls to take natural differences between color types into account. Another logical observation was that spleen size correlated positively with ELISA absorbances. ELISA absorbance also showed positive correlation with AMDV genome copy number in spleen (as copies/ng of DNA) and possible correlation with copy number in kidneys. This is similar to the earlier findings that genome copy number in blood was greater in the farm with a clinical course of infection as compared to the farm with subclinical infection (Kowalczyk et al., 2018). One mink with the clinical form of AD from farm 1 had clearly higher copy numbers in both tissues as compared to clinically healthy mink.

Transcriptome analysis revealed several up- or downregulated genes in infected mink as compared to non-infected mink and symptomatic mink as compared to non-symptomatic mink. These genes were involved not only directly in immune response but also other cellular processes. Many of the genes that were highly upregulated in symptomatic mink as compared to asymptomatic mink were related to innate immunity, which may partly be explained by the fact that farm 1 had been applying

selection for tolerant mink for several decades whereas farm 2 had only had AMDV for a couple of years and had not been actively selecting AMDV tolerant mink. The most significantly upregulated gene in mink from farm 2 (compared to farm 1) was *ONECUT2* ($\log_2FC=7.21$), which activates the transcription of several liver genes. Interestingly, Karimi et al. detected several genes involved in liver development to be strongly selected between groups of different disease severity (Karimi et al., 2021). Bloom et al. (1994), on the other hand, suggested a predominance of Th1 response (macrophage activation) over Th2 response (B cell activation and antibody production) in mink lacking the progressive disease. In addition to some proinflammatory genes, several genes involved in suppression of inflammatory response and activation of Th1 response were upregulated in farm 2. Also, considering the fact that farm 2 reported that the number of symptomatic mink was slowly decreasing, it appears that mink from farm 2 were also starting to tolerate the virus by suppressing inflammation and antibody production. It should be noted that some differences may have been caused by other factors than AMDV, including age, other infections, and possible differences in environmental conditions, but to minimize their effect, mink of same sex and color type were chosen, and the samples were collected at the same time of the year. These results give information about gene-level differences in mink with different AMDV status and help understand the mechanisms behind varying symptoms and immune response. Further studies, e.g., genome-wide association analysis and comparison of different color types, are needed to better understand the roles of environmental and genetic factors in AD severity.

One limitation to this study is the small sample size and the small number of farms. However, all the mink represented the same gender, excluding one male mink from farm 2. Limited sample size should also be taken into account when interpreting statistical tests. Especially with the non-significant correlations, results might have been different with a bigger sample size. Causes of death for the mink that died during the follow-up were also not reported, so it is not known if some of them died of AD. However, it is expected that some breeding females are lost during a long follow-up like this one for example due to nursing sickness. Even though decades of breeding and living with the virus is the most likely explanation for the different numbers of symptomatic mink in the farms, the effect of virus strain cannot be excluded either, as the two infected farms had different virus strains. More thorough analysis of virus sequences or complete genome sequencing was not conducted because frequent co-infections in farm 1 would affect the reliability of the sequencing results. However, the very diverse virus strains in farm 1 indicate the dominance of the host effect on different disease severity in farms.

5. Conclusions

This study provides a long-term follow-up on AMDV positive mink in farm conditions. It shows that mink can have normal production rates and be clinically healthy despite being infected with different strains of AMDV. After decades of breeding and living with AMDV, mink from farm 1 frequently had low antibody titers, low number of virus in tissues, and only mild histopathological changes from AD. A large proportion of ELISA negative but PCR positive mink raises the need to conduct further studies on antibody negative mink from farms with a different disease status to evaluate the reliability of antibody tests in diagnostics and disease control. Further studies are also needed to better understand the host factors behind disease tolerance to help select mink for breeding and avoid causing additional health issues by overbreeding.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.vetmic.2022.109452](https://doi.org/10.1016/j.vetmic.2022.109452).

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