Faculty of Veterinary Medicine
Department of Veterinary Biosciences
Faculty of Medicine
Department of Virology
Doctoral Program in Clinical Veterinary Medicine
University of Helsinki, Finland

EPIDEMIOLOGY AND PATHOGENICITY OF ALEUTIAN MINK DISEASE VIRUS

Jenni Virtanen

DOCTORAL DISSERTATION

To be presented for public discussion with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, in Hall 2, Haartman Institute, Haartmaninkatu 3, Helsinki, on May 20th, 2022, at 13 noon.
Helsinki 2022

Supervisors: **Associate Professor Tarja Sironen**
Departments of Virology and Veterinary Biosciences
Faculties of Medicine and Veterinary Medicine
University of Helsinki
Helsinki, Finland

Professor Olli Vapalahti
Departments of Virology and Veterinary Biosciences
Faculties of Medicine and Veterinary Medicine
University of Helsinki
Helsinki, Finland

Dr. Ravi Kant
Department of Veterinary Biosciences
Faculty of Veterinary Medicine
University of Helsinki
Helsinki, Finland

Pre-examiners: **Docent Petri Susi**
Institute of Biomedicine
University of Turku
Turku, Finland

Professor Per Saris
University of Helsinki
Helsinki, Finland

Opponent: **Dr. Marta Canuti**
University Of Milan
Milan, Italy

Custos: **Professor Olli Vapalahti**
Departments of Virology and Veterinary Biosciences
Faculties of Medicine and Veterinary Medicine
University of Helsinki
Helsinki, Finland

ISBN 978-951-51-8023-0 (paperback)
ISBN 978-951-51-8024-7 (PDF)
http://ethesis.helsinki.fi

Unigrafia, Helsinki University Print
Helsinki 2022

The Faculty of Veterinary Medicine uses the Ouriginal system (plagiarism recognition) to examine all doctoral dissertations.

ABSTRACT

Aleutian mink disease virus (AMDV), species *Carnivore amdoparvovirus 1* and family *Parvoviridae*, causes Aleutian disease (AD), one of the most significant infectious diseases of American mink. It causes high antibody levels, plasmacytosis, and immune complex disease with symptoms ranging from subclinical to fatal. After the first cases were reported in North America in the 1950s, the virus spread to all mink-producing countries and to nature, where it infected several other mustelid and carnivore species. This thesis focused on AMDV epidemiology, evolution, transmission, varying disease severity, and development of polymerase chain reaction (PCR) and sequencing methods to increase understanding on the disease and provide solutions for disease control.

Currently, AMDV diagnostics in Finland is based on antibody screening with ELISA and confirmation of positive results with PCR using DNA exctacted from spleen. However, non-specific products caused frequent delays in PCR diagnostics. To update the diagnostics, we validated two used SYBR green-based PCRs (pan-AMDV-PCR and pan-AMDO-PCR) and established a novel probe-based PCR (NS1-probe-PCR). All three PCRs had comparable sensitivities (down to 20 copies/reaction) defined by plasmid dilution series and diagnostic spleen samples. The NS1-probe-PCR was chosen as the primary method due to optimal performance.

We also optimized two previously published methods for whole-genome sequencing. Insufficient sequence data for an efficient primer design proved to be an issue with the first protocol relying on PCR amplification of the genome, which is why we modified a metagenomic approach for ssDNA virus sequencing and successfully used it to sequence the entire AMDV genome from five samples.

To study the impact of intense farming practices on evolution and transmission of AMDV, we analyzed virus strains from fur farms and free-ranging mustelids from Finland and Poland. Viral sequences indicated that AMDV had appeared in both countries on several separate occasions, and country-specific clustering and frequent transmission between countries were observed. Results from comparison of strains from farmed and free-ranging mink differed between countries. Sequence data from Poland confirmed that farming affects AMDV diversity in the wild within and between study sites.

Further sequence analysis identified frequent coinfection and recombination. Two major and several possible recombination breakpoints were identified. The evolution rate of AMDV was estimated to be higher than with most DNA viruses and slightly higher than with other parvoviruses, probably due to farming conditions with many mink being in close proximity. The degree of within-host evolution was higher in free-ranging mink than farmed mink, indicating that they had lived with the virus longer than farmed mink, which are usually culled annually. No notable difference was detected between free-ranging and farmed mink in positive and negative selection patterns in partial NS1 protein. However, a more thorough comparison would require a larger dataset of the complete genomes of both groups than is currently available.

American mink and native mustelids from Poland were analyzed for AMDV antibodies with ELISA. The proportion of infected mink was higher in males than females, adults than juveniles, and in areas close to the mink farms, indicating virus flow from farms into the wild. The proportion of ELISA-positive individuals among native mustelids appeared to correlate with the expected contact of the species with the mink. Almost all the ELISA-positive mink were PCR-positive, but only a fraction of the ELISA-positive native mustelids were also PCR-positive, suggesting lower persistence of AMDV in the latter.

In addition to being found among native mustelids, AMDV DNA was also detected in stools of farmed blue foxes, indicating that they may be infected and secrete the virus in stool. However, more research is required to exclude the possibility of viruses originating from the contaminated environment or food.

In order to study varying disease severity, we compared a farm with mainly asymptomatic mink and a decades-long history of breeding a disease-tolerant herd, a farm with symptomatic mink, and an AMDV-negative farm. Differences in viral loads in spleens and kidneys were observed between asymptomatic and symptomatic mink. In the farm with AMDV-tolerant mink, a notable proportion of ELISA-negative mink were PCR-positive during the 2.5-year follow-up, indicating very low antibody production. Transcriptome analysis revealed differences in gene expression between farms. The results show that mink can live with the virus for years without visible symptoms, have a normal litter size and pelt quality, and bring up breeding of tolerant herd as an option in disease control.

In summary, we updated PCR diagnostics to improve speed and reliability that are important in disease control. Additionally, we set up a metagenomic protocol to sequence AMDV without prior sequence information. These studies also brought new information about AMDV epidemiology and host dynamics that can be used to better understand virus history, and transmission between farms and the wild, between countries, and between species. Comparison of mink with a different disease status gave information about AMDV pathogenesis in different stages of infection and gave new insights into disease control.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and manuscript which are referred to in the text by their roman numerals:

I. **Virtanen J**, Smura T, Aaltonen K, Moisander-Jylhä A-M, Knuuttila A, Vapalahti O, Sironen T. 2019. Co-circulation of highly diverse Aleutian Mink Disease virus strains in Finland. J Gen Virol 100:227-236. doi:10.1099/jgv.0.001187

II. **Virtanen J**, Aaltonen K, Vapalahti O, Sironen T. 2019. Development and validation of nucleic acid tests to diagnose Aleutian mink disease virus. J Virol Methods 279:113776. doi: 10.1016/j.jviromet.2019.113776

III. Zalewski A, **Virtanen J**, Brzeziński M, Kołodziej-Sobocińska M, Jankow W, Sironen T. 2020. Aleutian mink disease: Spatio-temporal variation of prevalence and influence on the feral American mink. Transbound Emerg Dis 00:1–15. doi:10.1111/tbed.13928

IV. **Virtanen J**, Zalewski A, Kołodziej-Sobocińska M, Brzeziński M, Smura T, Sironen T. 2021. Diversity and transmission of Aleutian mink disease virus in feral and farmed American mink and native mustelids. Virus Evol. 7:1–12. doi:10.1093/ve/veab075

V. **Virtanen J**, Aatonen K, Moisander-Jylhä A-M, Nordgren H, Paulin L, Peura J, Vapalahti O, Kant R, Sironen T. Mechanisms behind the varying severity of Aleutian mink disease virus: comparison of three farms with a different disease status. Manuscript.

ABBREVIATIONS

aa	Amino acid
ADE	Antibody-dependent enhancement
AMDV	Aleutian mink disease virus
AD	Aleutian disease
bp	Base pair
BSA	Bovine serum albumin
CI	Confidence interval
CIEP	Counter-current immunoelectrophoresis
CPV	Canine parvovirus
DEG	Differentially exressed gene
GFAV	Gray fox amdovirus
IAT	Iodine agglutination test
kb	Kilobase
ssDNA	Single-stranded deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FPLV	Feline panleukopenia virus
LaAV	Labrador amdoparvovirus
MEV	Mink enteritis virus
NGS	Next generation sequencing
NS1	Non-structural protein 1
NS2	Non-structural protein 2
NS3	Non-structural protein 3
ORF	Open reading frame
PBS	Phosphate-buffered saline
PPV	Porcine parvovirus
PPE	Personal protective equipment
qPCR	Quantitative polymerase chain reaction
RFAV	Raccoon dog and fox amdoparvovirus
RFFAV	Red fox fecal amdoparvovirus
RpAPV	Red panda amdovirus
SKAV	Skunk amdoparvovirus
tMRCA	the most recent common ancestor
VP1	Structural protein 1
VP2	Structural protein 2

1 INTRODUCTION

1.1 AMERICAN MINK

The American mink (*Neovison vison*) is a medium-sized carnivore native to North America. It has also been introduced in the wild in Europe, South America, and Asia through fur farm escapees or intentional release [1]. Feral mink have spread to most European countries through farming or migration and are widely distributed in Poland, Belarus, Iceland, Denmark, and the Baltic countries [1]. The first reports of free-ranging mink in Finland occurred in 1932 [2]. In the 1950s, they were mainly found in western and southwestern parts of Finland but have since spread throughout the country. The presence of feral mink in Poland is due to migration through the eastern Polish–Belarusian border at the end of the 1970s [3] and from farm escapees, mainly in the western part of the country [4]. Currently, feral mink are spread throughout northern and central Poland [3]. American mink have negatively impacted native animal species such as nesting birds, rodents, polecats, European mink, amphibians, and crayfish through prey or competition [1].

American mink have been farmed for their fur since the late 1800s. Since then, several different coat colors have been bred, including mink with silver-greyish coat color (Aleutian mink) in the 1940s [5]. In Finland, the first fur farms were established in the 1930s [6]. The number of farms was highest in the 1980s (at over 5,000 farms) but has decreased since; approximately 700 farms were in operation in 2018. Farming is mainly concentrated in the Ostrobothnia region, which contains 95% of Finnish fur farms [7]. Between 2015–2020, the largest mink pelt producers were China, Denmark, and Poland, followed by the Netherlands, the USA, and numerous other countries with smaller production. However, the SARS-CoV-2 pandemic has led to permanent (e.g. the Netherlands) or temporary (e.g. Denmark) bans in several countries [8] (FIFUR Statistics 2021, email communication with Jussi Peura 3.11.2021).

1.2 PARVOVIRUSES

The family *Parvoviridae* was established in 1975 and divided into subfamilies *Parvovirinae* and *Densovirinae* in 1993*,* with viruses from *Parvovirinae* infecting vertebrates and those from *Densovirinae* infecting invertebrates. The classification was recently updated by splitting *Densovirinae* into the *Densovirinae* and *Hamaparvovirinae* subfamilies due to the discovery of vertebra-infecting parvoviruses more closely related to *Densovirinae* than *Parvovirinae [9, 10]*. *Parvovirinae* has 10 genera: *Amdoparvovirus, Artiparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Loriparvovirus, Protoparvovirus*, and *Tetraparvovirus* [9]. Parvoviruses cause diseases ranging from mild to severe in a wide range of mammals including humans, cats, dogs, cows, and rodents [11]. Some of the well-known pathogenic parvoviruses include human parvovirus B19, porcine parvovirus (PPV), canine parvovirus (CPV), feline panleukopenia virus (FPLV), and mink enteritis virus (MEV). New pathogenic

parvoviruses, such as equine parvovirus-hepatitis, tilapia parvovirus, and mouse kidney parvovirus, are regularly identified [12].

Parvoviruses have an icosahedral (T = 1) 23–28 nm non-enveloped virion and a linear, non-segmented ssDNA genome (4–6 kb). Most parvoviruses have two open reading frames (ORF): the left encodes non-structural proteins required for transcription and DNA replication, and the right encodes capsid proteins forming the virus capsid. Parvoviruses use one to three promoters and alternative splicing, alternative polyadenylation, and leaky scanning to obtain different gene products. Genome ends have terminal hairpin structures that act as primers in DNA replication, which occurs through a "rolling hairpin" mechanism. Parvoviruses replicate in the nucleus and require the cell to pass through the S-phase for replication, making young animals with a wide range of actively dividing cells particularly vulnerable [11].

1.3 ALEUTIAN MINK DISEASE VIRUS

1.3.1 AMDOPARVOVIRUSES

Aleutian mink disease virus (AMDV), originally detected in Aleutian mink, belongs to the genus *Amdoparvovirus* [13-15] and has been classified as species *Carnivore amdoparvovirus 1* [9]. It was the only member of the genus until 2011; however, more species have subsequently been identified. Based on the classification criteria, viruses within the same species are monophyletic. Their NS1 proteins are at least 85% similar in amino acid sequences, whereas the aa similarity of NS1 viruses within the same genus should generally be at least 35%–40% [10]. Other species of the genus in 2021 include *Carnivore amdoparvovirus 2* (gray fox amdovirus [GFAV] [16]), *Carnivore amdoparvovirus 3* (raccoon dog and fox amdoparvovirus [RFAV] [17]), *Carnivore amdoparvovirus 4* (skunk amdoparvovirus [SKAV] [18]), and *Carnivore amdoparvovirus 5* (red panda amdoparvovirus [RpAPV] [19]). Amdoparvoviruses also include unclassified Labrador amdoparvovirus, LaAV [20], and red fox fecal amdovirus (RFFAV) [21]. Another recently discovered raccoon dog amdoparvovirus (RDAM) is 94%–98% similar to RFAV in the NS1 aa sequence and hence can be classified as Carnivore amdoparvovirus 3 [22]. Some strains, such as those obtained from striped skunk by Nituch et al. [23], have been classified as AMDV despite being more closely related to other amdoparvovirus species (Figure 1). The NS1 aa similarity within Carnivore amdoparvovirus species 1–5 ranges from 65%–80%. In addition to AMDV, GFAV, RFAV, SKAV, and RpAV have been either shown or suggested to cause clinical disease in their host, although, in some cases, the connection between symptoms and the virus remains to be verified [16-19].

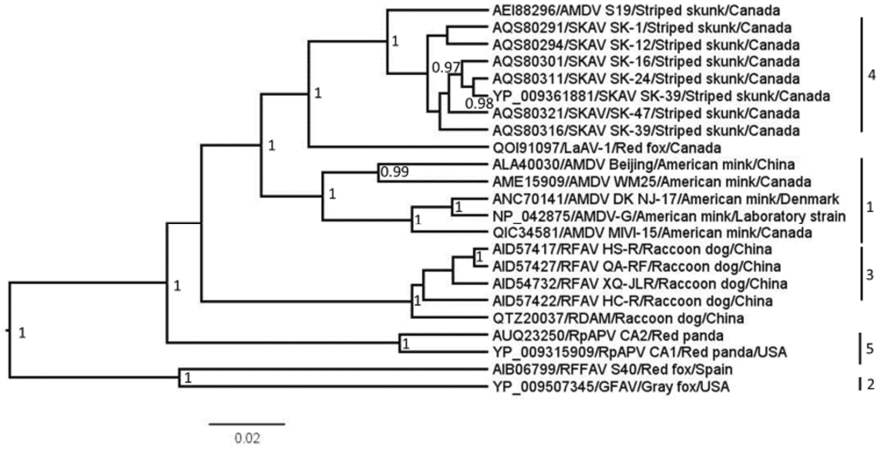

Figure 1 Amdoparvovirus phylogeny using partial VP2 amino acid sequence (aa 108–369) of representatives of all currently known species. The phylogenetic tree was built with Beast v2.4.7. The substitution model (WAG + G) was selected with MEGA6. Posterior probabilities above 0.9 are shown next to the nodes. Carnivore amdoparvovirus species 1–5 are numbered.

1.3.2 GENOME, PROTEINS, AND STRUCTURE

AMDV has a 4.8 bp ssDNA genome with predominantly negative polarity [24, 25]. A palindromic sequence at the 3′ end forms a Y-shaped structure, and the 5′ end has a terminal hairpin structure [26, 27]. Six mRNAs are produced from a single pre-mRNA by a P3 promoter at the left end of the genome processed by alternative splicing and alternative polyadenylation [28, 29].

AMDV has two ORFs encoding five proteins (Figure 2). The left ORF encodes non-structural proteins NS1 [30], NS2, and NS3 [28], and the right ORF encodes structural proteins VP1 and VP2 [25]. NS1 is located in the nucleus, contains DNA binding motifs and ATPase and helicase activities, and is essential for viral replication [27, 31]. NS2 is expressed at similar levels to NS1 and colocalized in the nucleus. NS3 is produced at lower levels and does not colocalize with NS1. However, both NS2 and NS3 are also crucial for virus replication [32]. VP1 and VP2 form a 23–26-nm virus capsid at a 1:9 ratio and 60 protein subunits [24, 25, 28, 33]. VP1 can also bind the 3′ terminus of viral DNA, leading to its encapsidation in virus capsids before its exit from the cells [34].

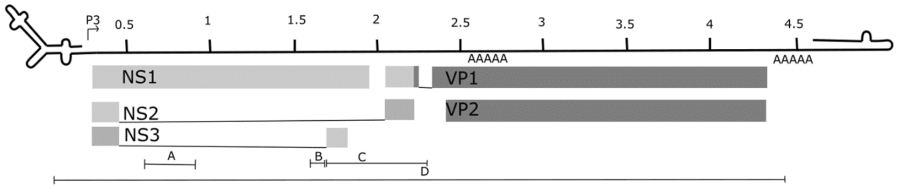

Figure 2 Representation of the AMDV genome including terminal hairpin structures at both ends, a P3 promoter, and polyadenylation sites (AAAAA). Regions encoding non-structural proteins are indicated with light gray boxes and structural proteins with dark gray boxes. Regions amplified by PCRs discussed in this thesis are marked A–D (A: pan-AMDV-PCR, B: NS1-probe-PCR, C: pan-AMDO-PCR, and D: entire coding region amplifying PCR).

1.3.3 CELL ENTRY AND REPLICATION

AMDV infects immune cells through antibody-dependent enhancement (ADE, Figure 3) [35, 36]. This is a phenomenon, first described for dengue virus, in which cellular FcγR-receptors recognize virus-antibody complexes, trigger a phagocytic pathway, and thus enhance virus entry into the cells [37]. With AMDV, at least the FcγRIIa-receptor (expressed on the surfaces of neutrophils, eosinophils, monocytes, macrophages, dendritic cells, platelets, and basophils in humans) has been recognized to mediate ADE [37, 38]. In adult mink, AMDV replicates in macrophages, B-lymphocytes, and dendritic cells in lymphoid organs (spleen and lymph nodes) [39-43]. Replication is restricted leading to persistent and non-cytopathic infection [40]. Permissive replication occurs in pneumocytes (alveolar type II cells) of seronegative mink kits [44-46]. AMDV VP1 lacks the phospholipase A2 motif that most parvoviruses use in escaping from endosomes, and the exact mechanism of cell entry remains partially unclear [47, 48]. In permissive replication, NS1 is cleaved from two sites by cellular caspases, facilitating its transport into the nucleus to regulate viral replication [47, 49, 50]. Cellular caspases have also been connected to maintaining persistent infection and restricting viral replication [51]. AMDV has proven challenging to cultivate, and only a few strains have been successfully adapted to cell culture [52]. The most widely used cell culture-adapted strain, AMDV-G, originates from the pathogenic Utah strain but has lost its pathogenicity after several passages in cells [25].

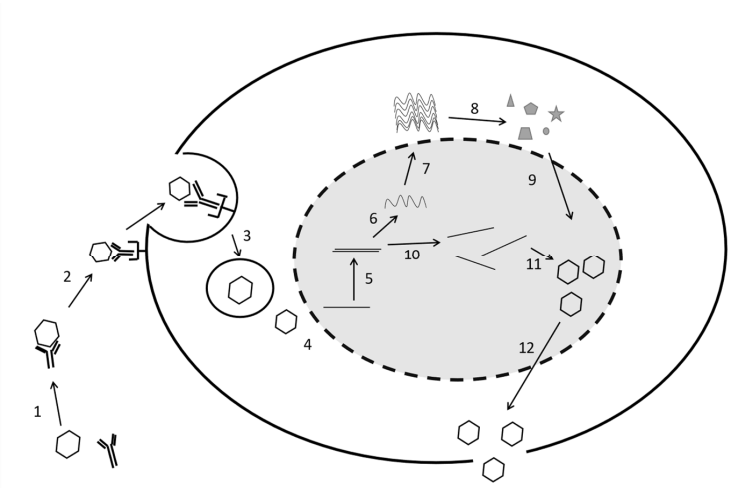

Figure 3 A simplified presentation of antibody-dependent enhancement and AMDV replication. 1: Formation of virus-antibody complexes; 2: Virus-antibody complexes bind to cellular Fc-receptors and enhance virus entry into the cell; 3: Endocytosis and transport into the nucleus; 4: Uncoating; 5: Conversion of ssDNA to dsDNA; 6: Transcription of pre-mRNA; 7: Generation of six mRNAs from pre-mRNA; 8: Translation of viral proteins; 9: Transport of viral proteins into a nucleus; 10: Replication of virus genome (ssDNA); 11: Virion formation; 12: Virus exit from cells. Figure is adapted from [47].

1.3.4 TRANSMISSION

AMDV can be transmitted horizontally, either directly or indirectly, through body fluids (blood, feces, urine, saliva) or contaminated environments [5, 53]. AMDV can also be transmitted to mink kits vertically through the placenta [54-56]. Airborne transmission has also been suggested, but its role in transmission is unclear [53]. AMDV, like parvoviruses in general, is extremely resistant to many standard chemical and physical treatments, including heat, pH 3, ether, fluorocarbon, deoxycholate, and protease and nuclease enzymes, making it difficult to eliminate the virus from infected farms to prevent the transmission. AMDV is inactivated by ultraviolet light, 0.05 N sodium hydroxide, 0.5 N hydrochloric acid, 4 N urea, and 0.5% iodine [14, 57-59].

AMDV is transmitted efficiently between farms. In addition to infected animals, contaminated equipment and visitors have been suggested to spread the virus between farms. AMDV DNA has been detected widely on surfaces in direct contact with infected animals (cages, gloves, personal protective equipment [PPE]) and in soil, streets, more distant surfaces of farms, and vehicles [60, 61]. Insufficient, poor-quality, and poorly used PPE increases transmission through people entering farms [62]. Previous infection on farms has been identified as a risk factor, probably due to the high stability of the virus in nature, which easily leads to insufficient inactivation [63, 64]. Larger farm sizes and infections in neighboring farms have also been identified as risk factors [65]. Vector-borne transmission through houseflies (*Fannia*

canicularis) [66], mosquitoes, [67] or ticks (*Haemaphysalis longicornis*) [68] has been suggested.

Virus transmission from farms to the wild has been noted, although the results have varied. Possible means of transmission from farms into the wild include farm escapees, direct contact between farmed mink and free-ranging animals, indirect contact through contaminated waste and premises, and unknown transmission mechanisms. Reported AMDV prevalences (antibodies or DNA) have varied from 0.00%–93.30% in free-ranging American mink [69]. In serological studies, Nituch et al. found evidence of mink farms being the source of the virus in free-ranging mink in Canada, although evidence pointed to a transmission route other than only farm escapees [70]. In genetic studies performed in Canada and Finland, strong evidence of virus transmission between farmed and feral mink was observed [20, 71, 72]. However, in Estonia and Poland, strains from feral and farmed mink were located in separate branches [73, 74]. Nevertheless, these studies were performed with an extremely limited number of samples. Based on phylogenetic analysis, AMDV has spread efficiently between farms from different countries despite efforts to prevent this, although country-specific clustering was also detected [75-79]. The viral population size expanded until the 1990s, when more effective control measures were applied [79].

1.3.5 HOST RANGE

In addition to American mink, evidence of natural AMDV infections has been detected in several other mustelid species (Table 1), such as ferrets (*Mustela furo*), European mink (*Mustela lutreola*), stone marten (*Martes foina*), pine marten (*Martes martes*), pacific marten (*Martes caurina*), stoat (*Mustela erminea*), river otter (*Lontra canadensis*), Eurasian otter (*Lutra lutra*), and European badger (*Meles meles*) [74, 80-85]. Other carnivore species with natural infections include red fox (*Vulpes vulpes*), bobcat (*Lynx rufus*), striped skunk (*Mephitis mephitis*), common genet (*Genetta genetta*), and raccoon (*Procyon lotor*) [20, 23, 82, 83, 86-91]. Of these, ferrets and possibly skunks have developed symptoms [85, 88, 92, 93]. However, in some cases, the results have not been confirmed with sequence analysis, and the possibility of a positive result being another amdoparvovirus cannot be excluded. For example, some of the results about AMDV in skunks were published before a very similar SKAV was established and may be produced by SKAV misclassified as AMDV. Allender et al. reported that the partial capsid protein sequence from symptomatic skunk was slightly distinct from other AMDV strains, and strains found by Glueckert et al. were slightly closer to SKAV than AMDV. However, the lack of longer sequences has prevented more accurate characterization [18, 86, 92]. Natural AMDV infection has been detected in none of the tested rodents (Beaver [*Castor canadensis*], muskrat [*Ondatra zibethicus*], groundhog [*Marmota monax*], and red squirrel [*Tamiasciurus hudsonicus*]) [82, 87].

Concerns have been expressed about possible danger to wildlife. It was suggested that AMDV might be causing the decline in the European mink population [80, 83]. It has also been suggested that mink is the maintenance host for AMDV and is required for virus persistence in the population, although transmission between species is common [84].

Regarding experimental infections, ferrets, stoats, fishers, American martens, raccoon dogs, raccoons, cats, dogs, blue foxes, mice, and rabbits have developed antibody response against AMDV. However, evidence of viral replication has only been found in ferrets, raccoons, raccoon dogs, and dogs. Infectious virus has also been detected in mice and cats, and only ferrets and possibly striped skunks developed histologic lesions [85, 89, 94, 95].

Reports in humans are rare, considering the extensive exposure of many individuals to mink. Aleutian disease (AD) was first suspected in humans in the 1960s in patients with a disease having clinical signs resembling mink AD. However, these were not confirmed by antibody tests or PCR [6]. Possible AMDV antibodies have been reported in exposed but asymptomatic laboratory workers and unexposed hospital patients. However, the possibility of cross-reaction with other parvoviruses could not be excluded [96]. AMDV antibodies and DNA have also been found in two mink farmers, one of whom had arteritis and the other glomerulonephritis [97]. Further confirmation is required to determine whether AMDV has zoonotic potential.

Table 1. *Prevalence of AMDV antibodies and DNA in free-ranging animals from different studies. The number of positive animals per number of tested animals is reported in parentheses.*

Species	Antibodies	Virus DNA	Protocol	Country	Collection time	Reference
American mink	0% (0/14)	20% (2/5)	CIEP, PCR	Spain	1997–1999	[80]
	55% (16/29)	N/A	Unknown	Canada	Unknown	[87]
	52% (14/27)	N/A	CIEP	Southern England	Unknown	[98]
	46% (65/142)	58% (80/139)	ELISA and PCR	Sweden	2004–2009	[99]
	N/A	79% (11/14)	PCR	Canada, NS	2006–2008	[100]
	23% (17/75)	N/A	CIEP and CCLAI	France	Unknown	[83]
	N/A	25% (45/183)	PCR	Canada, O	2005–2009	[72]
	3% (13/296)	N/A	CIEP	Denmark, mainland	1998–2009	[101]
	45% (64/142)	N/A	CIEP	Denmark, Bornholm	1998–2009	[101]
	N/A	42% (32/77)	PCR	Canada, BC	2002–2019	[84]
	93% (56/60)[a]		CIEP, PCR	Canada, NS	2009–2011	[82]
	32% (562/1735)	N/A	CIEP	Spain	1997–2012	[81]
	54% (31/57)[a]		ELISA, PCR	Finland	2006–2014	[71]
	N/A	72% (34/47)	PCR	Canada, N	2014–2016	[20]
	29% (60/208)	N/A	CIEP	Canada, O	2005–2009	[70]
	N/A	24% (39/164)	PCR	Iceland	2010–2018	[102]
	55% (30/55)	N/A	CIEP	Canada	Unknown	[103]
	N/A	4/27	PCR	Estonia	2007–2010	[82]
European mink	33% (3/9)	100% (1/1)	CIEP, PCR	Spain	1997–1999	[80]
	13% (12/99)	N/A	CIEP and CCLAI	France	Unknown	[83]
	32% (150/410)	N/A	CIEP	Spain	1997–2012	[81]
	0% (0/84)	N/A	CIEP	Spain	2004–2005	[104]
	N/A	0% (0/4)	PCR	Estonia	2007–2010	[74]

Species	Antibodies	Virus DNA	Protocol	Country	Collection time	Reference
Stone marten	24% (4/17)	N/A	CIEP and CCLAI	France	Unknown	[83]
	N/A	0% (0/1)	PCR	Estonia	2007–2010	[74]
Pine marten	6% (1/16)	N/A	CIEP and CCLAI	France	Unknown	[83]
	0% (0/183) [a]		ELISA, PCR	Finland	2006–2014	[71]
	N/A	0% (0/49)	PCR	Estonia	2007–2010	[74]
Polecat	11% (16/145)	N/A	CIEP and CCLAI	France	Unknown	[83]
	7% (1/14) [a]		ELISA, PCR	Finland	2006–2014	[71]
	N/A	0% (0/42)	PCR	Estonia	2007–2010	[74]
Pacific marten	N/A	3% (4/130)	PCR	Canada, BC	2002–2019	[84]
Fisher	0% (0/47)	N/A	Unknown	Canada	Unknown	[87]
	0% (0/6) [a]		CIEP, PCR	Canada, NS	2009–2011	[82]
	N/A	0% (0/38)	PCR	Canada	Unknown	[86]
Stoat	71% (43/61) [a]		CIEP, PCR	Canada, NS	2009–2011	[82]
	N/A	0/27, 0%	PCR	Canada, BC	2002–2019	[84]
	0% (0/1) [a]		ELISA, PCR	Finland	2006–2014	[71]
	N/A	0% (0/17)	PCR	Canada, N	Unknown	[20]
Least weasel	0% (0/2) [a]		ELISA, PCR	Finland	2006–2014	[71]
River otter	18% (2/11) [a]		CIEP, PCR	Canada, NS	2009–2011	[82]
	0% (0/59)	0% (0/59)	CIEP, PCR	Canada, O	2011–2012	[105]
	N/A	0% (0/22)	PCR	Canada, BC	2002–2019	[84]
Eurasian otter	N/A	100% (1/1)	CIEP, PCR	Spain	1997–1999	[80]
	0% (0/24) [a]		ELISA, PCR	Finland	2006–2014	[71]
	N/A	0% (0/2)	PCR	Estonia	2007–2010	[74]
European badger	27% (7/26) [a]		ELISA, PCR	Finland	2006–2014	[71]
	N/A	0% (0/4)	PCR	Estonia	2007–2010	[74]
Wolverine	0% (0/1) [a]		ELISA, PCR	Finland	2006–2014	[71]
Red fox	2% (2/100)	N/A	Unknown	Canada	Unknown	[87]
	0% (0/25) [a]		CIEP, PCR	Canada, NS	2009–2011	[82]
	N/A	6% (3/50)	PCR	Canada, N	2012–2018	[20]
Arctic fox	N/A	0% (0/1)	PCR	Canada, Labrador	2018	[20]
Coyote	0/5, 0%	N/A	Unknown	Canada	Unknown	[87]
	0% (0/24) [a]		CIEP, PCR	Canada, NS	2009–2011	[82]
	N/A	0% (0/87)	PCR	Canada, N	2014–2016	[20]
Raccoon dog	N/A	0% (0/23)	PCR	Estonia	2007–2010	[74]
Lynx	N/A	4% (2/58)	PCR	Canada, N	2012–2017	[20]
Bob cat	10% (2/20) [a]		CIEP, PCR	Canada, NS	2009–2011	[82]
Striped skunk	65% (128/196)	N/A	Unknown	Canada	Unknown	[87]
	25% (2/8) [a]		CIEP, PCR	Canada, NS	2009–2011	[82]
	41% (143/347)	32% (14/40) [b]	CIEP, PCR	Canada, O	2006–2008	[23]
	N/A	86% (43/50) [b]	PCR	Canada, BS	2011–2015	[90]
	N/A	65% (140/216) [c]	PCR	Canada	Unknown	[86]

Species	Antibodies	Virus DNA	Protocol	Country	Collection time	Reference
Raccoon	4% (1/27)	N/A	Unknown	Canada	Unknown	[87]
	11% (9/85) [a]		CIEP, PCR	Canada, NS	2009–2011	[82]
	0% (0/144)	N/A	CIEP	Canada, O	2006–2008	[23]
Common genet	4% (3/68)	N/A	CIEP and CCLAI	France	Unknown	[83]
Beaver	0% (0/58) [a]		CIEP, PCR	Canada, NS	2009–2011	[82]
Muskrat	0% (0/59) [a]		CIEP, PCR	Canada, NS	2009–2011	[82]
Groundhog	0% (0/7)	N/A	Unknown	Canada	Unknown	[87]
Red squirrel	0% (0/45) [a]		CIEP, PCR	Canada, NS	2009–2011	[82]

[a] Either antibody or PCR-positive

[b] May not be AMDV due to PCR also detecting some SKAV strains

[c] Classified as AMDV but is closer to SKAV (see strain AMDV S19, Figure 1). Table includes the results that were classified as AMDV in the original publications.

CIEP = counter-current immunoelectrophoresis, PCR = polymerase chain reaction, CCLAI = countercurrent line absorption immunoelectrophoresis, O = Ontario, BC = British Columbia, NS = Nova Scotia, N = Newfoundland

1.3.6 EVOLUTIONARY RATE AND RECOMBINATION EVENTS IN PARVOVIRUSES

Generally, RNA viruses tend to have faster mutation rates than DNA viruses, although many exceptions exist [106]. In addition to AMDV, many parvoviruses, such as CPV and FPLV, are characterized by a high substitution rate closer to that of RNA viruses [79, 106-108]. Indications also exist that CPV may have a higher substitution rate in the capsid protein than FPLV, although NS1 rates were similar [107]. The predominance of negative compared to positive selection in NS1 and VP2 has been noted in AMDV and other parvoviruses. A higher percentage of positively selected sites in NS1 compared to VP2 has also been observed for AMDV [109-113], which might partly explain the unusually high diversity in the NS1 region compared to other parvoviruses [23, 109]. Homology modeling has located most of the sites under diversifying selection in NS1 on the protein surface [79]. The low level of positive selection in the capsid protein sequence may be explained by a continuous availability of new individuals to infect and AMDV using the host immune response, so that escaping the host immune response is not beneficial for the virus [109]. Recombination can be frequent in parvoviruses and it has been detected with PPV, mouse parvoviruses, minute viruses of mice, LuIII virus, hamster parvovirus [114], CPV, FPLV, and MEV [115, 116]. With AMDV, breakpoints have been detected at approximately 900 bp from the beginning of NS1, around the middle of the genome, and in the middle and end of VP2 [109, 114].

1.4 ALEUTIAN DISEASE

1.4.1 HISTORY

AD was first identified in 1940s in North America in mink with the Aleutian genotype. The virus had probably been circulating in mink previously. However, it had remained undetected until a disease-susceptible Aleutian mink was bred in 1941 [5]. Viral etiology was first suggested in the early 1960s, and parvovirus was suggested as a causative agent in the 1970s [13, 15, 24, 117]. The disease was reported in Sweden and Denmark in the 1950s and 1960s and has subsequently spread to all mink-producing countries and into the wild [6]. The virus origin has been estimated as the late 18th century, possibly in some non-mink wild species [79].

1.4.2 CLINICAL SIGNS AND IMMUNE RESPONSES

Clinical signs of AD range from subclinical to severe and fatal. In adults, clinical signs include anorexia; low-quality fur; weight loss; pale, enlarged, and mottled kidneys; enlarged spleen, liver, and mesenteric lymph nodes; plasmacytosis (a high proportion of plasma cells in tissues), hypergammaglobulinemia; necrotizing arteritis; neurological symptoms; mouth ulcers; reproductive failure; and glomerulonephritis [118]. Severe disease is characterized by a massive number of antibodies and immune complexes that accumulate in tissues, causing tissue damage and lesions [119]. Disease outcomes in adult mink can be roughly classified as follows: 1) progressive infection with high antibody titers, hypergammaglobulinemia, lesions, and high mortality; 2) persistent, non-progressive infection with low antibody titers, no lesions, and little or no hypergammaglobulinemia; and 3) transient infection with clearing of the virus [120]. In kits, AD manifests as acute interstitial pneumonia caused by a cytopathic effect in pneumocytes. Transfer of maternal antibodies protects from pneumonia and restricts viral replication in pneumocytes but does not prevent the chronic adult form of the disease [44, 45, 121].

Experimental infections to determine the progression of the immune response have detected significant elevations in the amount of $CD8^+$ lymphocytes for at least 6 months [122, 123]. Antibodies are produced against structural and non-structural proteins [30, 124]. They cannot neutralize the virus [119], and no immunity to protect from future infections by another strain is achieved [125]. The IgM response has been detected around a week after infection and peaks at 2–3 weeks, after which it slowly subsides. Low levels of AMDV-specific IgA are also found [126]. Most of the produced antibodies are IgG antibodies, which can usually be detected 2–3 weeks post-infection despite much variation depending on experimental conditions, virus dose, mink genotype, and virus strains; moreover, individual variation has been detected [53, 78, 119, 126-128]. Generally, Aleutian mink tend to have somewhat higher IgG levels against AMDV than non-Aleutian mink. However, transiently high antibody levels have also been detected in asymptomatic non-Aleutian mink [126, 129]. Among feral mink, AMDV antibodies are more common in adults than juveniles, probably due to the longer possible exposure [83, 98, 99]. In experimental infections, the time of detectable viremia in blood based on PCR has also varied from a few days to several weeks post-infection [127, 130].

1.4.3 PATHOGENESIS

The varying severity of the disease has been attributed to host and virus factors. Virus strains may vary from highly pathogenic, often causing severe disease in Aleutian and non-Aleutian mink, to low pathogenic, causing severe disease mainly in Aleutian mink [120, 131]. Comparative analysis of capsid protein sequences has been used to identify regions and aa residues playing a role in pathogenesis, and differences between pathogenic strains and non-pathogenic AMDV-G have often been confined to a relatively small number of aa residues [27, 89]. For example, in a comparison of brown mink from two farms with clinical and subclinical courses of infection, only one amino acid difference with the potential to affect the functionality of the protein was detected in the VP2 sequence [132].

In addition to the virus factors, the clinical picture is linked to mink age and genotype. AD was originally detected in mink homozygous recessive for the Aleutian gene (e.g., sapphire and violet) but subsequently also in other genotypes. Mink with the Aleutian genotype appear more susceptible to the disease, possibly due to Chediak-Higashi syndrome, a disorder caused by a mutation in the *lysosomal trafficking regulator* gene (*LYST*) and characterized by diluted pigmentation and susceptibility to infections [118, 133-135]. It has been estimated that up to a quarter of non-Aleutian mink can clear the virus [56, 136]. Genomic regions containing genes involved in virus–host interactions, immune responses, reproduction, and liver development have been recognized as being subject to selection for a response to AMDV infection [137].

1.4.4 DIAGNOSTICS

In addition to analyzing clinical signs and gross and histopathological lesions, several methods have been developed to diagnose AMDV. The first diagnostic tests developed in the 1960s and 1970s were mainly based on detecting increased globulin levels (iodine agglutination test [IAT], serum electrophoresis, and glutaraldehyde test). The IAT was widely used in diagnostics; however, it had poor specificity and sensitivity, identifying only symptomatic mink in the later stages of infection. Hence, it was insufficient to eradicate AMDV from farms [5, 52]. Immunohistochemical testing of AMDV antigen, and a wide range of serological tests have also been developed, of which CIEP and ELISA were mainly used due to their simplicity and rapidity. PCR-based methods are also used in diagnostics in confirmatory testing but cannot be applied on a larger scale due to complexity and costs [5, 6, 138].

CIEP has been used in most mink-producing countries for large-scale screening for decades. It was also used in Finland from the 1980s to 2008 but has been replaced by ELISA due to the latter's good specificity and sensitivity, lower cost, and good large-scale applicability [6, 139, 140]. Currently, hundreds of thousands of mink are screened with ELISA annually. Unclear cases and those from previously clean farms are then confirmed with PCR tests from spleen tissue at the University of Helsinki, as the spleen is the most persistently infected tissue [53]. Until 2020, a combination of pan-AMDO- and pan-AMDV-PCRs [71, 138] was used. However, insufficient specificity required frequently confirming the results with Sanger sequencing, prolonging the time required to reach the diagnosis.

1.4.5 TREATMENT, PREVENTION AND DISEASE CONTROL

No effective treatment or vaccine is in use despite several attempts to develop one. Vaccines based on inactivated virus or capsid protein and treatment with passive antibodies have been found to be ineffective or even exacerbate the disease [141-143]. Vaccines based on non-structural proteins have led to partial protection from the disease but have not prevented infection [47, 141, 144, 145]. Immunosuppressed mink treated with cyclophosphamide developed no lesions, and mink treated with immunostimulating methisoprinol had fewer virus copies in the spleen and lymph nodes than control mink [146, 147]. In a recent study, VP2 aptamers inhibited half the AMDV production in infected cells in vitro. However, they have not yet been tested in vivo [148]. However, costs make most of these treatments unsuitable for larger-scale application on mink farms.

Different countries have used varying control strategies, including testing, culling of infected animals, disinfection, and trade and visitor restrictions. In Finland, no mandatory control program has been established by the government, but the Finnish Fur Breeders' Association has implemented an AMDV eradication program based on regular antibody screening. The annual mean seroprevalence in tested farmed mink varied between 3% and 60% in 1980–2014, being highest when the testing began in the 1980s [6] and 27% in 2020 (M. Eerola [FIFUR], personal communication 20.10.2021). In Denmark, a voluntary AMDV sampling and testing policy was implemented in 1976 and legislated in 1999, leading to a generally low prevalence (5%) from 2001–2015. However, after 2015, several outbreaks of unknown origin were detected [63, 149]. A selective AMDV control program was implemented in Poland; based on analysis of an extremely limited number of farms, the AMDV prevalence was higher on farms that had not implemented the program [150]. In China, where no national-level program has been established, the average prevalence has increased from 48% in 1981–2009 to 61% in 2010–2017 [151]. Spatial differences have also been reported in China and Denmark [63, 151, 152]. In Iceland, AMDV was successfully eradicated from a few remaining farms in the 1980s, excluding one case in 1996, through annual testing of all farms, culling of mink from infected farms, and thorough disinfection after an outbreak [153]. However, information on prevalence on farms from many countries is scarce and often reliant on voluntary testing and reports.

Breeding has been recognized as a tool in disease control. In Nova Scotia, Canada, an efficient testing and removal strategy appeared to reduce the proportion of infected mink but was often insufficient to eradicate the virus. Several farms have applied selection of AD-tolerant mink as a form of disease control using health status, litter size, fur quality, and IAT results and achieved litter sizes and mortality rates comparable to AMDV-free farms. Breeding has also been shown to reduce the severity of histopathological lesions in AMDV-positive mink herds [154, 155]. Selecting mink with low antibody titers for breeding on infected farms has also been suggested [137, 156].

2 AIMS OF THE THESIS

The main goal of this doctoral thesis was to study the epidemiology, pathogenicity, and host tolerance of AMDV and deepen understanding of different AMDV strains, their transmission, and effects on American mink and native mustelids. There were the following specific aims:

- To gain information about AMDV diversity and transmission between and within countries, between farms and the wild, and between host species by sequencing virus strains circulating in Finland and Poland (I and IV*)*;

- To increase understanding of AMDV evolution and dynamics in different host species (I, III, and IV);

- To illuminate how the disease tolerance varies by comparing mink of different color types and mink from two farms with different disease histories (V);

- To develop and optimize a robust PCR method for AMDV diagnosis.

3 MATERIALS AND METHODS

3.1 SAMPLES

Study I used 52 AMDV-positive DNA samples from the spleen of farmed mink from 24 farms in Finland and 44 AMDV-positive DNA samples from free-ranging mustelids in Finland and Estonia. Samples from farmed mink were originally collected for diagnostic purposes between 2015 and 2017 by Fin FurLab, and samples from free-ranging mustelids were from a study by Knuuttila et al. [71]. Diagnostic PCR protocols (Study II) were validated with a set of 46 DNA samples from the spleens of farmed mink also collected for diagnostic purposes between 2016–2019 and 37 CPV, FPLV, or MEV-positive stool samples from mink, blue foxes (*Vulpes lagopus*), and a raccoon dog (*Nyctereutes procyonoides*) originally collected for another project.

For studies III and IV, the carcasses of 1,153 feral mink, 60 farmed mink, and 152 native mustelids were collected between 2006–2017 (Figure 1, studies III and IV). Feral mink were collected from nine study sites (Białowieża Forest [BF], Biebrza National Park [BNP], Narew National Park [NNP], Vistula River [VR], Gwda River [GR], Drawa National Park [DNP], Warta Mouth National Park [WMNP], Słowiński National Park [SNP], and Modła Lake and surrounding area [ML]), which were further combined into three regions (west [WMNP, DNP, GR], east [VR, NNP, DNP, BF], and north [SNP, ML]). Farmed mink were sampled from three farms in northwestern Poland, and martens, otters, and polecats were all collected in the eastern part of the country. Most mink farms (54%) are in the western region as compared to the northern (17%) and eastern (29%) regions. This study was conducted in collaboration with Professor Andrzej Zalewski from the Mammal Research Institute, Polish Academy of Sciences. Sampling and weight and age data collection were managed by our collaborators.

Samples for Study V were collected from three farms. Farm 1 had struggled with AMDV for decades and controlled it by breeding weak antibody-positive but asymptomatic animals and reported rarely having mink with clinical disease. Farm 2 had been infected for less than 5 years and reported having mink with AD symptoms, and Farm 3 was clean. Blood samples on filter paper and rectal swabs were taken from Farm 1 on four occasions between 2017–2019. The experiment started with 30 mink (white, brown, and sapphire), 13 of which died of unreported causes during the follow-up. Five white mink with signs of anorexia and dehydration and a positive ELISA test in a screening a few weeks prior were sampled from Farm 2, and seven healthy white mink were sampled from Farm 3. Serum (BD Vacutainer) and blood samples (Tempus blood RNA tubes, Applied Biosystems) were taken with a cardiac puncture under terminal anesthesia from all the mink. The health of the mink before sampling was assessed, samples were taken, and autopsies were performed by a veterinarian.

No ethical permissions were mandatory for these projects, as samples were originally collected for diagnostic purposes (spleen samples of studies I and II); sampling was non-invasive (studies II and V); or samples were collected from

euthanized, dead, or trapped animals (studies I, III, IV, and V). Mink for studies III–IV were eradicated as part of nature protection plans, killed by hunters, or collected as roadkill.

3.2 DNA EXTRACTION (II, IV, AND V)

A NucleoSpin tissue kit (Macherey-Nagel) was used for DNA extraction from tissue and blood samples. DNA was extracted from tissues with a standard protocol for tissue samples (II, IV, and V) and from blood by incubating a 1 cm^2 piece of filter paper in 300 µl of PBS o/n at 4°C and using a support protocol for viral DNA from blood samples for extraction (V). DNA from stool samples was extracted either with a QIAamp Fast DNA Stool Mini Kit (Qiagen) or with a QIAQube HT and DNeasy 96 PowerSoil Pro QIAcube HT Kit (V).

3.3 PCR AND SEQUENCING METHODS

3.3.1 REAL-TIME PCR PROTOCOLS AND SANGER SEQUENCING (I, II, IV, AND V)

SYBR green-based pan-AMVD-PCR amplifying the region 578–951 (partial NS1; all the genomic regions in this thesis are according to strain AMDV-G [GenBank accession no. M20036.1]) was modified from PCR by Jensen et al. [138] (details in Study I). SYBR green-based pan-AMDO-PCR amplifying the region 1662–2302 (partial NS1 and NS2) was described by Knuuttila et al. [71]. Probe-based NS1-probe-PCR (details in Study II) was established for Study II and was designed to amplify 1586–1645 bp of all known strains published in GenBank by March 2019. PCRs were performed with Stratagene Mx3005P (Agilent Technologies). Pan-AMDV- and pan-AMDO-PCR products were sequenced by purifying them with a GeneJET PCR purification kit (I) or by adding 0.5 µl of Exonuclease I and 1 µl of FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) to 5 µl of PCR reaction and by incubating at 37°C for 45 min and 85°C for 15 min (II, IV, and V), after which they were sequenced with Sanger sequencing.

3.3.2 WHOLE CODING REGION SEQUENCING BASED ON PCR AMPLICONS (I)

The entire coding region (nt 79–4467) amplifying PCR was performed for all samples of Study I with primers from Hagberg et al. [157], either in one part with primers AMDVF1 + AMDVR3 (4,390 bp) or in two parts with primers AMDVF1 + AMDVR2 (3,240 bp) and AMDVF2 + AMDVR3 (3,022 bp). Primers were modified to match all the sequences published in GenBank by 2016. PCR products were sequenced with MiSeq (Illumina). Gaps in the genome were amplified by PCR and sequenced with Sanger sequencing. All samples of wild mink from which the whole coding region was successfully sequenced and six randomly selected farm samples were tested for coinfection by amplifying nt 578–951 by PCR, cloning it into PGEM-T Vector System I (Promega), and sequencing 7–10 clones with Sanger sequencing. Protocol details are described in Study I.

3.3.3 WHOLE GENOME SEQUENCING BASED ON METAGENOMICS (IV)

A metagenomic protocol established for fecal samples [158] was modified to sequence AMDV from tissues. The general outline of the protocol is presented in Figure 4, and the details are described in Study IV.

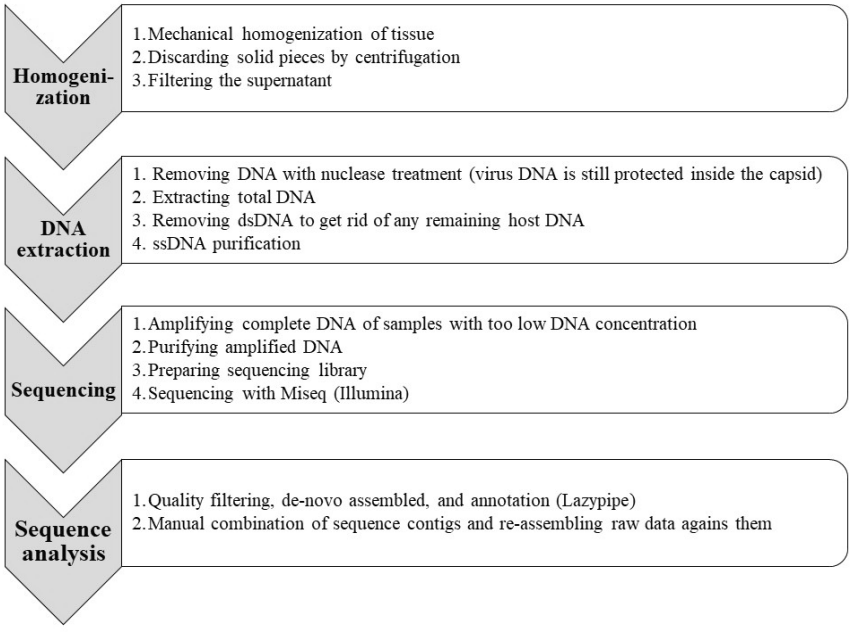

Figure 4 Outline of the metagenomic sequencing protocol to sequence ssDNA viruses from tissue samples.

Table 2. *Primer sequences*

Primer or probe	Sequence	Position in AMDV-G	Reference
AMDVF1	CGC TTC GCG CTT GCT AAC TTC	79–97	[157]
AMDVF2	CTA TGT ACC ATC CTA ACC AAG CAA RGT	1445–1471	[157]
AMDVR2	GCA RAG AGG AGG TAG MCC CAA[a]	3297–3317	[157]
AMDVR3	TTA ATC CGC CAC TTT CTG GT	4448–4467	[157]
AMDV754	GTG GAT TGA AGA TGG GCC	754–771	Study I
AMDV1005	GCT TTC TTC CCA GTC TAT ATC AG	1005–1027	Study I
AMDV1256	CTT GAA MTR TCT TGG GAA CCA	1256–1276	Study I
AMDV1526	TGC TTT ACA TAT TAA RGA TGC TAG	1526–1549	Study I
AMDV2439	GAG CAA GCA ACT AAC CAA ACT	2439–2459	Study I
AMDV2633	CCA GCA TTA AAT ACT AAG TAT TCA	2633–2656	Study I
D_AMDV F 7 H PN1	CAT ATT CAC TGT TGC TTA GGT TA	578–600	[138]
D_AMDV R 7 H PN2	CGT TCT TTG TTA GTT AGG TTG TC	929–951	[138]
D_pan-AMDO F	AAG ACT TTA AAG CCA TTA CTG GA	1662–1684	[71]
D_pan-AMDO R	GGA TAG TGC TGA GGA ACT AAA G	2281–2302	[71]
AMDV NS1 F2	GGA AAR ACC YTR CTR GCA TCY YT	1154–1536	Study II
AMDV NS1 R	GTT ACC RCA CTC TTC ASH CC	1626–1645	Study II
AMDV NS1 probe	6-FAM-AAC TTT CCA TGG ACT GA-MGB	1586–1645	Study II

[a] Slightly modified from the reference

3.3.4 TRANSCRIPTOME SEQUENCING (V)

RNA for the transcriptome analysis (V) was extracted from blood samples collected in Tempus blood RNA tubes from three mink (white females) from each farm with a Tempus™ Spin RNA Isolation Kit (Applied Biosystems), and quality were checked with Bioanalyzer (Agilent). Transcriptomes (single-end reads) were sequenced with NextSeq (Illumina) at DNA Sequencing and genomics lab (Institute of Biotechnology, University of Helsinki).

3.4 VALIDATION OF REAL-TIME PCR PROTOCOLS (II)

Analytical sensitivities of pan-AMDV-, pan-AMDO-, and NS1-probe-PCRs were determined with plasmids containing the PCR products amplified from an AMDV-positive mink (MN590274-MN590276, plasmid preparation detailed in Study II). All three PCRs were performed for dilution series of 1,000, 100, 50, 20, 10, 5, 1, and 0 copies/reaction of plasmids in five parallel reactions. One parallel reaction containing 4 µl of DNA from an AMDV-negative mink spleen was also included to check whether the host DNA affected sensitivity. Diagnostic sensitivities and specificities for each PCR were tested with the spleen and stool samples described earlier. The results were analyzed based on Ct values, dissociation curves, by running the products

in 2% agarose gel, or by Sanger sequencing to determine whether the signal was due to positive or non-specific results. PCRs giving contradictory results were repeated.

3.5 ANTIBODY TESTS (III–V)

Antibodies against AMDV were tested with AMDV VP2 ELISA [140]. The ELISA cut-offs for each antigen lot, conjugate, and sample material were determined by testing a panel of negative samples and adding two standard deviations to the mean absorbance. Blood from the heart or spleen of mustelids from Poland (III and IV) was first absorbed by filter paper strips air-dried and stored at −20°C. Filter paper samples (III–V) were analyzed from a circular piece (5 mm) incubated o/n in 100 µl of dilution buffer (PBS + 0.5% BSA + 0.05% Tween 20) and used in ELISA. Serum samples were diluted at 1:200. Goat anti-ferret IgG (H + L) secondary antibody (Novus) with 1:20,000 dilution or peroxidase-conjugated AffiniPure Goat Anti-Cat IgG (H + L; Jackson ImmunoResearch) at 1:4,500 dilution was used as a conjugate. All blood and serum samples from studies III–V were analyzed with ELISA, and all ELISA-positive native mustelids from studies III–IV were subjected to PCR. Due to the large number of samples, representative mink were selected for PCR analysis by selecting 20 ELISA-positive individuals (or all of them if the number was less than 20) with simple random sampling without replacement from each study site.

3.6 PATHOLOGY AND HISTOLOGY (III AND V)

Mink from Study III were measured (body mass and body length without tail) to estimate body condition index, and heart, liver, kidneys, and spleen were collected. The relative weights of the liver, kidneys, and spleen to the body mass were calculated. Age was estimated based on dental data as described in Project III. This part was performed by our collaborators.

All mink from Farm 1 and two of the mink from Farm 3 (Study V) were submitted for necropsy for gross and histopathological examinations performed by a veterinary pathologist. Briefly, the body weight and weight of the spleen were obtained, and brain, heart, lungs, spleen, liver, kidney, intestines, and other tissues with possible abnormalities based on macroscopic evaluation were obtained in formalin for histology. Samples from the spleen and kidney were also taken for PCR. Lesions and macroscopic changes were scored from 0–3.

3.7 DATA ANALYSIS (I, II, IV, AND V)

3.7.1 SEQUENCE ASSEMBLY AND ANALYSIS (I, II, IV, AND V)

Sanger sequence raw data were analyzed with MEGA6 [159]. Next generation sequencing (NGS) raw data from PCR products (I) was assembled by mapping reads against the reference sequence (AMDV-G) with UGENE [160] using Bowtie 2 [161] and checked using BWA-MEM 0.7.17 [162]. Raw NGS data of PCR-free sequencing (IV) was assembled with Trimmomatic [163], Megahit [164], and SANSparallel [165] in the Lazypipe pipeline [166]; contigs were combined manually; and the raw sequence reads were reassembled to the consensus sequence using Bowtie 2.

MEGA6 was used to align the sequences with Clustal W [167] (I, II, and V) or Muscle [168] (IV) to define the best evolutionary models and calculate p-distances with pairwise deletion. Recombination analysis was performed on all alignments with RDP [169], GENECONV [170], BootScan [171], Max-Chi [172], Chimaera [173], SiScan [174], and 3Seq [175] of RDP v4.92 (I) or RDP5 (IV) [176]. Recombination events recognized by at least four programs (with a p-value of 0.05) were removed from the alignment before further analysis, and those recognized by at least five programs were analyzed in more detail. Intra-host variation (Study I) was estimated by filtering the raw date with Trimmomatics, assembling it with BWA-MEM and removing PCR duplicates with SAMtools 1.8 [177, 178], and calling the single nucleotide variants with LoFreq 2 [179]. Positive and negative selection in the pan-AMDV region (aa 145–244 of NS1, Project IV) was analyzed for farmed and feral strains from Poland (Study IV and GenBank) with single-likelihood ancestor counting (SLAC), fixed-effect likelihood (FEL), mixed effects model of evolution (MEME), and fast, unconstrained Bayesian approximation for inferring selection (FUBAR) programs available online (www.datamonkey.org) [180-183]. Sites recognized by at least two methods were considered.

Reads from transcriptome analysis were trimmed with Trimmomatic [163], quality checked with FastQC [184], and aligned to the mink (Neovison vison GCF_020171115.1) reference genome with Bowtie 2 [161], followed by counting aligned reads per genes using HTSeq [185]. Differentially expressed genes (DEGs) were searched with edgeR (p-value cut-off 0.05) [186]. All the analyses was conducted in R.

Bayesian phylogeny for Projects I, IV, and V was performed with Beast v1.8.2 [187], Tracer v1.6 [188], and FigTree v1.4.2 [189], and only trees with all effective sample size values above 100 were accepted. IQ-trees (IV) were constructed with version 2.1.2 [190] using ModelFinder [191] and ultrafast bootstrapping [192]. The sequences acquired during this thesis were deposited to GenBank with accession numbers MG821234-MG821259 and MG821261-MG821309 (I), MN590274-MN590284 (II), MZ126964-MZ127162 (IV), and OM142153-OM142203 (V). Detailed parameters are included in the figure legends (trees included in the theses) or in corresponding publications (trees only included in original publications).

3.7.2 STATISTICAL TESTS

For comparison of two or more groups, the data distribution was first estimated using the Shapiro-Wilk test. If the data was not normally distributed ($p < 0.05$), non-parametric tests were used. Tests were conducted with IBM Statistics 24 (I) and 27 (IV and V). Diagnostic sensitivities and specificities were calculated with MedCalc software (https://www.medcalc.org/calc/diagnostic_test.php, last accessed 26th October 2021; II).

Statistical analysis for AMDV seroprevalence in wild mustelids in Poland (III–IV) was conducted by our collaborators in Poland and is detailed in Study III. Briefly, all feral mink were analyzed with a generalized linear model (GLM) using sex, season (breeding [February–August] and non-breeding [September–January]), age (adult or juvenile), and region (west, east, or north) as explanatory variables, and wild mustelids in eastern Poland were analyzed with species, sex, and season as explanatory variables. Temporal variation in seroprevalence in feral mink in relation to the number of farmed mink was analyzed with generalized additive models (GAMs) using collection year, number of farmed mink within a 60-km radius from site center, sex, age, and season as explanatory variables. GAM analysis was performed for 2009–2016 samples from WMNP (high farming intensity) and NNP and BNP (low farming intensity) using age, sex, season, and year as explanatory variables to test the differences between temporal dynamics on sites with different farming intensities. The connection between AMDV infection and the body condition of mink was determined by regressing body mass on body length and spleen, kidney, and liver sizes on body mass and using the residuals as the response variable and ELISA result, sex, age, and season as explanatory variables in a simple linear model. Correlations between genomic and geographic distances (IV) were estimated with the Mantel test in R v4.0.3.

4 RESULTS AND DISCUSSION

This section summarizes the results and discussion from the four published articles and one manuscript for which this thesis is based on. A more detailed description of the results and discussion is presented in each article.

4.1 PROTOCOL DEVELOPMENT AND OPTIMIZATION

One of the goals was to develop and update PCR and sequencing protocols for diagnostics and research purposes. Firstly, the SYBR green-based pan-AMDV- (Study I) and pan-AMDO-PCRs [71] previously used in diagnostics had not been properly validated and had issues with non-specific products from the host genome, resulting in a frequent need to confirm the results by sequencing, which prolonged diagnosis by days. To solve the specificity issue and avoid time-consuming sequencing in diagnostics, we designed a new probe-based PCR. Secondly, the growing number of new amdoparvoviruses discovered in recent years also raised questions concerning the suitability of current PCR protocols for detecting them. Pan-AMDO-PCR had been designed to amplify other amdoparvoviruses; however, it was established when only GFAV and RFAV had been discovered and had not been checked for newly discovered amdoparvoviruses. The third reason for the requirement for protocol development was the growing need to sequence complete genomes to obtain a more comprehensive and reliable picture of virus evolution now that evolving sequencing protocols are making whole-genome sequencing cheaper and more accessible. Metagenomic protocols have been applied to other amdoparvoviruses [19, 21, 22], but the sequencing of complete genomes of AMDV from animals has generally relied on PCR amplification and the palindromic sequences in genome ends have rarely been sequenced [20, 84, 109, 157, 193-195].

4.1.1 VALIDATION OF PAN-AMDV-, PAN-AMDO-, AND NS1-PROBE-PCRS (II)

The analytical sensitivity of pan-AMDV-, pan-AMDO-, and NS1-probe-PCRs was tested with a plasmid dilution series, giving all PCRs a sensitivity of 20 copies/reaction. An additional plasmid dilution series containing DNA from AMDV-negative mink spleen was used to detect whether clinical spleen samples have an inhibitory effect in PCR. Differences in Ct values were less than one cycle, meaning no notable inhibitory effect was observed. The specificity for AMDV was tested with a set of stool samples known to contain other related parvoviruses (MEV, FPLV, or CPV), and no cross-reactivity was detected. However, some non-specific products

from the host sequence or other microbiota were observed, especially for pan-AMDO-PCR based on Sanger sequencing and a nucleotide BLAST search.

Diagnostic specificities and sensitivities (Table 3) were calculated by considering samples positive if they were positive with at least one of the PCRs (the results of individual samples are presented in Tables 1 and 2 in Study II). Pan-AMDV- and pan-AMDO results were considered false positives if they gave a signal in PCR, but only the host sequence was acquired in sequencing, and NS1-probe-PCR was considered false positive if the Ct value exceeded 42.0. Due to the limited sample sizes, differences of only a few percentage points were not considered reliable. No major differences were detected in the diagnostic sensitivity of PCRs of spleen samples. The specificity of NS1-probe-PCR for spleen samples may have been influenced by one sample with a Ct value of 44 being considered false positive despite possibly being true positive. This sample was positive in initial diagnostics with pan-AMDV- and pan-AMDO-PCR (data not shown) but had since been subjected to several freeze-and-thaw cycles, which probably explains its negative results in this study. NS1-probe-PCR had the best specificity with stool samples, whereas pan-AMDO-PCR had poor specificity with both sample materials.

Table 3. *Specificities and sensitivities of spleen samples and all samples combined as percentages. 95% confidence intervals are reported in brackets. Modified from Table 3 in Study II.*

		NS1-probe-PCR	**pan-AMDV-PCR**	**pan-AMDO-PCR**
Spleen samples	Specificity	88.9* [51.8–99.7]	100.0 [66.4–100.0]	44.4 [13.7–78.8]
	Sensitivity	97.3 [85.8–99.9]	94.6 [81.8–99.3]	94.6 [81.8–99.3]
All samples	Specificity	97.5 [86.8–99.9]	87.5 [73.2–95.8]	15.0 [5.7–29.8]
	Sensitivity	90.7 [77.9–97.4]	93.0 [80.9–98.4]	88.4 [74.9–96.1]

* If an unclear sample with Ct value 44.03 is excluded, the specificity is 100%

The primer sequences were compared against other amdoparvoviruses included in Figure 1 to obtain updated information about the probability of PCRs working for other amdoparvoviruses (Table 4). Pan-AMDO-PCR primers generally matched well with other viruses of the same genus, excluding one to two mismatches in the centers of the primers. However, the suitability of pan-AMDV- and NS1-probe-PCRs for detecting other amdoparvoviruses is highly uncertain.

Table 4. *Similarity of primers and probe of pan-AMDV-, pan-AMDO-, and NS1-probe-PCRs against other amdoparvoviruses used in Figure 1 (shown as identical nucleotides/length of the primer as base pairs). None of the mismatches were at the ends of the primers.*

	pan-AMDV PCR		**pan-AMDO PCR**		**NS1 probe PCR**		
	Forward primer	Reverse primer	Forward primer	Reverse primer	Forward primer	Reverse primer	Probe
GFAV	19/23	17/23	22/23	22/22	19/23	17/20	16/17
RFAV	23/23	17–18/23	23/23	21–22/22	21–22/23	17/20	16/17
RDAM	23/23	17/23	23/23	22/22	21/22	17/20	16/17
SKAV	23/23	19–20/23	22–23/23	22/22	22–23/23	18–20/20	17/17
RpAPV	21–22/23	17/23	21/23	22/22	19/23	19/20	16/17

Based on these results, none of the three PCRs can be considered superior to the others, and the PCR protocols should be selected based on the intended use. As probe-based NS1-probe-PCR proved to have comparable sensitivity with spleen samples and good specificity compared to the two SYBR green-based PCRs (Table 3), it was adopted for use in diagnostics after this project to enable faster and easier results without need for time consuming confirmations with gel run and sequencing. Pan-AMDV- and pan-AMDO-PCRs remain in scientific use in situations where sequence data from the virus strains is also required, as the region amplified by pan-AMDV-PCR is commonly used in phylogenetic analysis and has a comprehensive set of sequence data available in GenBank.

4.1.2 WHOLE-GENOME SEQUENCING PROTOCOLS (I AND IV)

Two different protocols were optimized and used to sequence the entire AMDV coding region. The first protocol was modified from a method established by Hagberg et al. [157] and relied on amplification of the coding region by PCR and sequencing of the PCR products with NGS. To sequence all the different virus strains, we updated the primers to match all the complete coding regions published in GenBank in 2016. With this protocol, we successfully sequenced the entire coding region from 31/97 AMDV-positive samples from Finland (nine free-ranging mink and 22 farmed mink, Study I), 1,500 bp onwards from three free-ranging mink, and fragments from one farmed mink and one pine marten. The most likely explanations for the unsuccessful whole-genome sequencing of the remainder of the samples are freeze-and-thaw cycles and mismatches with the primers, as only a few sequences from the genome ends were available for primer design. Primer mismatches are supported by the strains not successfully sequenced by this PCR generally being located in the same branches in phylogenetic trees based on shorter sequences acquired by pan-AMDV- and pan-AMDO-PCRs. If the whole-genome sequencing was unsuccessful, pan-AMDV- and pan-AMDO-PCRs were successfully used to sequence 17 additional farm samples. Partial genomes of strains from free-ranging animals were not sequenced, as this had already been conducted by Knuuttila et al. [71] and the sequences are available in GenBank.

To solve the issue with primer mismatches, we modified the metagenomic protocol established by Conceicao-Neto et al. for Study IV to sequence AMDV with no prior sequence information [158]. We sequenced the complete or nearly complete genomes from five heart tissue samples from Poland (four feral mink and one farmed mink). All these samples had strong signals in pan-AMDV-PCR, were placed in different branches in a phylogenetic tree, and had a clear Sanger sequence that did not indicate coinfection, which is why they were selected. Two strains (158/NV/DPN/2010 and 869/NV/WMNP/2014) were sequenced from nt 3–4560 and 151–4560. Nearly complete genomes were acquired from three other samples (11/NV/Farm/2009, 151/NV/BNP/2010, and 1049/NV/NNP/2014), excluding a few randomly distributed gaps of a few to a couple of hundred nucleotides.

Sequencing new or diverse viruses can be challenging, as most of the commonly used and cheapest protocols rely on specific PCRs; thus, prior knowledge about the virus sequence is required. For amdoparvoviruses, several new viruses have been identified from wild animals in recent years, highlighting the need for PCR-free sequencing approaches. Sequencing viruses directly from tissues is challenging, and our initial sequencing attempts without the step of removing dsDNA were unsuccessful due to the large amount of host DNA (data not shown), although similar protocols have been successfully used for other amdoparvoviruses, such as RDAM, RFFAV, and RpAV. Differences may be caused by different sample materials, smaller amounts of virus in the samples, or small differences in the workflow. Adding dsDNA removal to the workflow to remove host DNA remaining in the sample after nuclease treatment helped solve the problem. As only strong positive samples were sequenced in this thesis, the sensitivity of the protocol is unknown; however, it can be assumed to be weaker than that of PCR-based protocols and may require further optimization for samples with lower virus amounts. As the dsDNA is removed during this process, the protocol may be applied to the sequencing of any ssDNA viruses from tissues and thus should also be suitable for sequencing of other parvoviruses and ssDNA viruses, many of which are human or veterinary pathogens and a possible threat to companion and wild animals. However, this remains to be tested.

4.2 AMDV TRANSMISSION AND EVOLUTION

4.2.1 GLOBAL AMDV PHYLOGENETICS AND TRANSMISSION IN FINLAND

One of the main aims of this thesis project was to study virus transmission and evolution in Finland and globally. This was accomplished by analyzing phylogenetic trees comprising all sequenced virus strains (from Study I and GenBank published by June 2018) from a partial NS1 coding region (nt 578–951, pan-AMDV-PCR; Figure 5), nt 1662–2302 (pan-AMDO-PCR, Figure S3 in online supplementary material of Study I), and a partial VP coding region (nt 2417–3413; Figure 2B of Study I). The two regions within NS1 were used for practical reasons, as PCR protocols for these were readily available, and nt 578–951 has a large amount of sequence data in GenBank (see Figure 2 for locations of the PCRs in the genome). Nt 2417–3413 was selected to represent the capsid protein sequence due to the relatively good set of sequence data in GenBank and the lack of recombination detected by RDP. Nt 578–951 is characterized by high genetic variability, whereas the variability in nt 2417–3413 is smaller, probably because it is not beneficial for the virus to escape the host immune response by changing the capsid protein [79]. In all three phylogenetic trees, the Finnish strains could be found in most major branches rather than a single country-specific cluster, supporting the observations regarding frequent virus transmission between countries [75] and multiple introductions to Finland over the decades [112]. Based on the molecular clock analysis performed on nt 2417–3413, the smallest estimated separation time between Finnish and published foreign strains was 16 years (95% HDP = 9–28 years) between strains from Finland and Denmark, indicating no virus transmission to or from Finland within the last few years. However, transmission cannot be excluded either, as many of the circulating strains may not have been sequenced and published. Regarding transmission between farms and the wild, the smallest most recent common ancestor (tMRCA) between a Finnish farm and a free-ranging strain was 18 years (95% HDP = 12–30 years), indicating transmission between farms and the wild during the years. No evidence of recent transmission (within a few years) was found. However, this may be partly explained by sampling biases, as many of the free-ranging strains originate from areas with a low farming frequency. It is also noteworthy that tMRCA values should be considered only as estimates, indicating that the time might be shorter or longer than estimated.

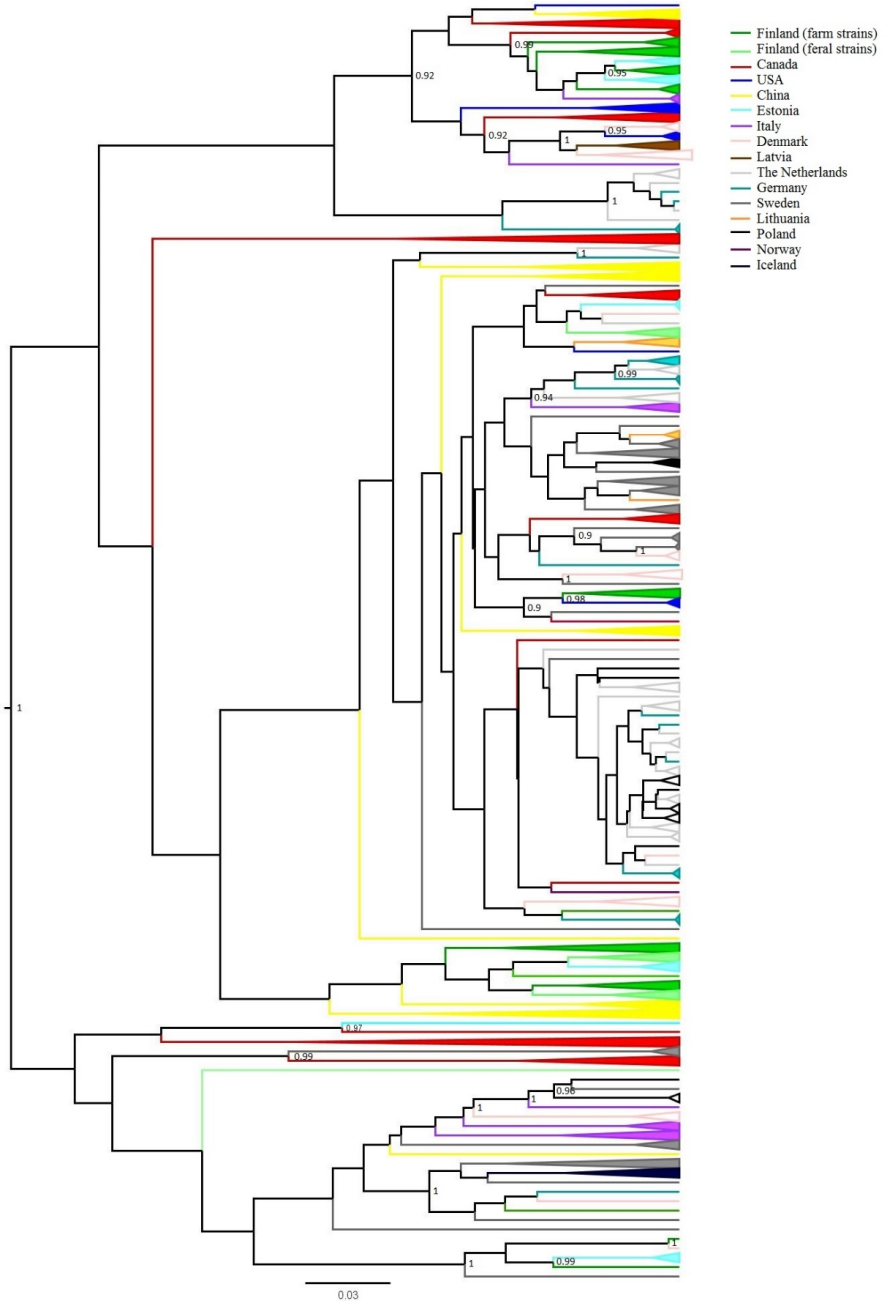

Figure 5 Global phylogeny of AMDV based on partial NS1 sequences (nt 578–951) published in GenBank by June 2018. Posterior probabilities above 0.9 are shown next to the nodes, and sequences from different countries are color-coded. Figure 2A of Study I.

4.2.2 AMDV TRANSMISSION AND DIVERSITY IN POLAND

In further investigating AMDV among wild animals and the effect of intense farming practices on virus dynamics, AMDV diversity, evolution, and transmission were also analyzed with a dataset of wild mustelids from Poland, where most mink farming is concentrated in the western part of the country. Phylogenetic analysis was again conducted on nt 578–951 and nt 1662–2302 (simplified trees are provided in Figure 3 in Study IV and uncondensed trees in its online supplementary material), and nt 578–951 was also analyzed with a molecular clock (Figure 2 in online supplementary material of Study IV). After excluding poor-quality sequences, 87 sequences (78 feral and five farmed mink, one polecat, and three stone martens) from nt 578–951 and 104 sequences (98 wild mink, four farmed mink, one pine marten, and one stone marten) from nt 1662–2302 were usable for further analysis.

Generally, the genetic mean distance in wild mustelids was highest in eastern Poland and lowest in western Poland, although within-region variation in study sites was also noted (Figure 6). A mild positive correlation was observed between genetic and geographic distances (Figure 4 in Study IV) between study sites in nt 578–951 ($R = 0.104$ and $p = 0.042$ in Mantel test) but not in 1662–2302 ($R = -0.026$ and $p = 0.068$ in Mantel test), which might be partly explained by different genetic variability (overall mean p-distance of all sequences used in phylogenetic analysis was 0.101 in nt 578–951 and 0.078 in nt 1662–2302). While the geographic distance may affect genetic distance, a relatively small correlation between the two factors and intriguing outliers (e.g., higher genetic diversity between study sites located close together in eastern Poland compared to western Poland) indicate that other aspects play a role in AMDV diversity and transmission. These may include geographical factors not considered in the analysis, different farming intensities in eastern and western Poland leading to different numbers of farm escapees attempting to establish themselves in the wild, and different introduction routes of feral mink on different sides of the country (farm escapees vs. migration of wild mink across borders).

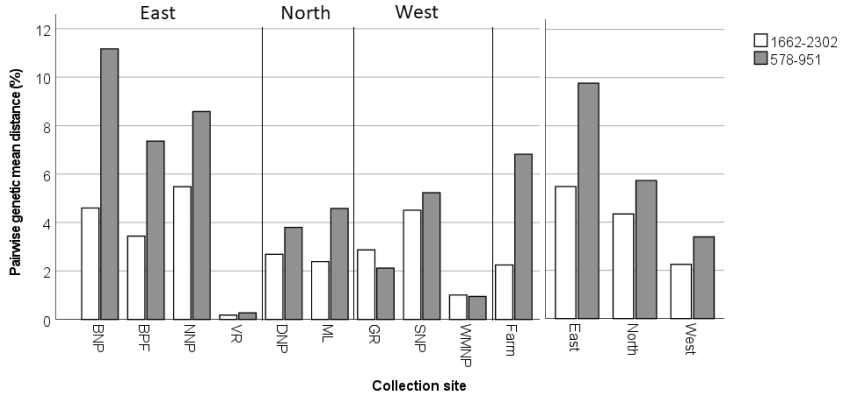

Figure 6 Within-group mean distances of wild mink in Poland using Sanger sequencing data from nt 578–951 and nt 1662–2302.

As in Finland, AMDV strains in Poland also formed several separate clusters in phylogenetic trees, indicating multiple introductions into the country through farm escapees or migration of wild animals. Some within-country geographic clustering was noted, and most of the recently emerged clusters had an identifiable main geographic region, although frequent mixing was also detected, especially when considering the older clusters. Incongruencies were found in the tree topologies between the two genomic regions, and several strains were clustered differently between trees. Possible reasons for different tree topologies include recombination and nt 1662–2302 being more conserved than nt 578–951. Despite these incongruences, both trees showed similar results concerning the overall movements of the virus and indicated spread of the virus between study sites over the decades.

Most farmed strains sequenced in this study grouped with previously published farmed strains from the Greater Poland Voivodeship. Sequences from farmed mink were located separately from feral strains and, in most cases, were more closely related to farmed strains from other European countries. This indicates the predominance of transmission between farms, for example, through trade, over transmission between farms and the wild. Our results are consistent with those of a previous comparison of farmed and feral strains in Poland [73] but differ from those of similar comparisons performed in Canada, where feral strains were similar to the strains found on local farms [84]. Sampling biases may be one reason for the lack of genetic evidence of virus transmission between farms and the wild. For example, farms willing to provide samples for research may have fewer mink escapees than farms that do not participate in research. Different conditions between farms and the wild should also be considered. Such differences include denser host populations and more frequent animal changes due to annual culling and virus eradication attempts on farms. Pathogens such as AMDV may also affect farm escapees' chances of survival in the wild, reducing the probability of farm strains being established in the wild. One limitation in our comparison of farm and feral strains is that the data set mainly relied on farm sequences from other studies. However, those sequences have been collected from the same regions during the same time frame, making them suitable for analysis.

Phylogenetic analysis was also conducted on complete genomes of this study and GenBank after removing strains with possible recombination (Figure 5 in Study IV). The results largely support those of partial genomes concerning the overall movements of the virus. Performing the analysis with complete genomes may increase the phylogenetic resolution [195]. However, due to the small and biased data set of complete genomes available in GenBank and the higher costs of whole-genome sequencing, which decrease the number of samples that can be sequenced, whole genome analysis brought minimal added value to phylogenetic analysis of this study, where sequence data was required from a huge number of samples.

4.2.3 WITHIN-HOST VARIATION (I)

Complete genomes of AMDV from Finnish mink were used to analyze within-host variation (Figure 7), which can result from either one virus strain evolving during chronic infection or from coinfection by several virus strains. One sample (W249) showed clear signs of coinfection by two different virus strains (nine sequenced clones located in separate branches in Figure S1 in online supplementary material of Study I) in molecular cloning and was excluded from all the analysis. Single nucleotide polymorphism within the host in the complete coding region was higher for the strains from free-ranging mink (2.11×10^{-3}) than from farmed mink (5.52×10^{-4}; Mann-Whitney U-test, $p < 0.001$). One probable explanation is that free-ranging mink may have carried the virus longer than farmed animals, which are usually culled annually, giving the virus more time to evolve. Coinfections caused by low-copy-number virus strains cannot be excluded either despite the lack of evidence in cloning. The variant frequency of farmed strains was higher in the NS coding region (nt 206–2211) than in the structural region (nt 2206–4349; Wilcoxon signed-rank test, $p = 0.006$). However, the difference was not statistically significant with free-ranging strains (Wilcoxon signed-rank test, $p = 1.000$). Different results between farmed and free-ranging strains may be caused by either higher negative selection in the VP region or more positive selection in the NS region among farm animals. Previous studies that detected more frequent positive selection in the NS region than the VP region did not separate farmed and wild animals [109].

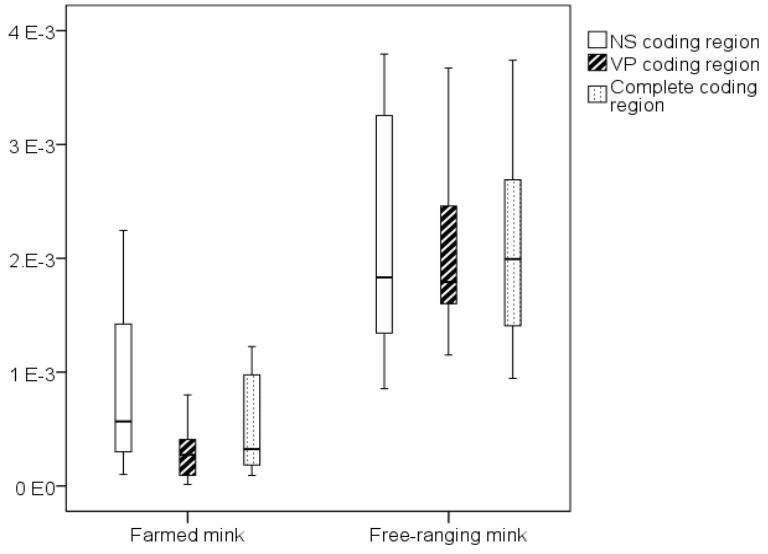

Figure 7 Variation within samples. Box-plot presentation of variant frequencies calculated from complete coding sequence, NS coding region, and VP coding region from strains of farmed mink and free-ranging mink. Modified from Figure 2A in online supplementary material of Study I.

4.2.4 RECOMBINATION ANALYSIS

Previous studies have detected recombination within AMDV and other parvoviruses [109, 114, 116, 196]. As no immunity is acquired to protect from further infections by other strains, coinfections are common especially among farmed mink living in crowded environments [109]. This high likelihood of coinfection might lead to recombination which can complicate assessing virus movements and relationships between strains, especially as different studies often use different regions for analysis. In this thesis, phylogenetic analysis was performed on two or three different genomic regions to determine whether they support the same overall conclusions about AMDV movements. Complete coding sequences from Finland (I) and Poland (IV) were analyzed to study the extent of recombination and identify common breakpoints. When recombination events recognized by at least five programs implemented in RDP were considered, possible recombination was identified in 14/30 complete genomes from Finland in seven events (Study I) and 2/5 complete genomes from Poland in two events (Study IV). Phylogenetic analysis of three different regions from the same dataset (Figure 8) and grouping scan analysis of selected recombination events (Study I) revealed major differences in tree structures and supported several of the recombination events suggested by RDP (e.g., events 1 and 4). Based on RDP and grouping scan analysis, major recombination breakpoints were located at approximately nt 1000 and nt 2000 (similar to the breakpoints identified by Canuti et al. [109]), and several less clear but possible breakpoints were found elsewhere in the genome. Further analysis of the recombinants of sequences from Poland was less clear, and the strains may not have been actual recombinants, but only divergent strains or parental strains being recombinants (detailed analysis can be found in Study IV and its online supplementary material). Although the exact proportion of recombinant strains was difficult to estimate due to possible false positives and the lack of information on many parental strains, it was clear that recombination was not uncommon. Whether the events occurred on farms or in the wild was unclear, as many of them may have occurred decades ago.

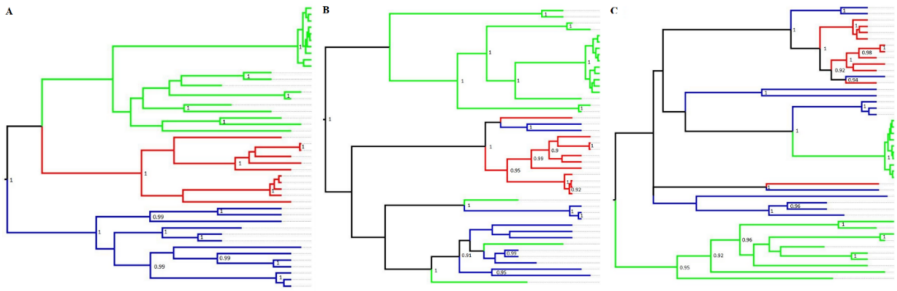

Figure 8 Phylogenetic comparison of different regions of AMDV genome using all complete coding regions from Study I and GenBank published by June 2018. Trees were constructed from the regions 1–700 (A), 1165–1864 (B), and 2446–3443 (C) using Beast v1.8.0 with Bayesian Skyline, lognormal relaxed clock, and 50,000,000 chain length. Best fitting evolutionary models (GTR + G for A and B and HKY + G + I for C) were defined using MEGA6. Posterior probabilities above 0.9 are shown next to the nodes, and branches are color-coded based on clusters in tree A to visualize differences in tree topologies. Simplified from Figure 1 of Study I. Strain names and grouping scans of selected recombinant strains are in the original publication.

Table 5. *Recombination analysis of complete genomes from Studies I (RDP4) and IV (RDP5). Recombination events recognized by at least five programs are included.*

	Beginning	Ending	Recombinant	RDP	GENECONV	BootScan	Max-Chi	Chimaera	SiScan	3Seq
I	UN	4054	F13	1.30E−16	1.99E−13	1.49E−14	2.24E−10	1.58E−11	5.38E−17	1.21E−17
	1135	1923	W454	6.89E−13	1.56E−08	NS	6.69E−08	7.30E−09	1.18E−12	6.04E−12
	431	1939	F2, F3, and F5	5.62E−04	NS	NS	3.64E−04	4.85E−06	1.38E−12	2.30E−09
	1869	UN	W181	5.34E−03	4.26E−03	NS	3.50E−06	6.19E−05	2.40E−08	NS
	3305	UN	F6, F12, F1, F14, F15, F7	2.99E−04	2.19E−04	2.95E−04	2.23E−04	5.83E−05	5.45E−07	7.25E−04
	1017	1851	W456, W458	6.16E−04	NS	NS	4.48E−04	1.86E−04	3.63E−04	1.33E−03
	UN	UN	W456, W458	2.10E−02	NS	1.47E−02	1.75E−03	8.76E−05	9.66E−04	3.96E−04
IV	1907	UN	151/NV/BNP/2010	NS	8.23E-06	2.87E-12	1.12E-11	8.15E-11	3.50E-17	6.22E-09
	2988	UN	1049/NV/NNP/2014	NS	NS	4.96E-03	1.45E-05	1.85E-02	6.27E-08	.024

NS: not significant at the level of 0.05

UN: undetermined

4.2.5 EVOLUTION RATE AND SELECTION PATTERNS

The mean evolution rate of AMDV was compared to those of other viruses. Based on complete coding sequences used in Figure 8, the estimated substitution rate (substitutions/site/year) was 3.39×10^{-3} in nt 1–700 (95% HPD 1.82×10^{-3}–5.00×10^{-3}), 4.478×10^{-3} (95% HPD 2.4571×10^{-3}–6.7893×10^{-3}) in nt 1165–1864, and 4.47×10^{-3} (95% HPD 2.46×10^{-3}–6.79×10^{-3}) in nt 2446–3443. It was 9.05×10^{-4} (95% HPD 4.34×10^{-4}–1.38×10^{-3}) in 2417–3413 (Figure 2B in Study I). Variation

in the evolution rate between branches was also observed. The observed substitution rates are somewhat higher than those of CPV and FLPV (generally around 10^{-4}–10^{-5} [106, 108]) and other ssDNA viruses, which may be explained by intense farming practices that can lead to different selection pressures and more frequent virus transmission between animals.

Around the time these results were published, another study noted differences in the selective force distribution between wild and domestic mink in NS1 sequence [79]. Due to the differences in terms of lifespan and proximity of other mink on farms and in the wild, we compared the selection patterns of a partial NS1 sequence (aa 145–244) between feral and farmed mink using a large amount of sequence data from Poland. In farmed strains, 5% of codons were positively selected and 7% negatively selected. In feral strains, 6% were positively selected and 12% negatively selected (Table 6). The direction of selection was generally similar between the two groups, although the strength of the selection differed. Consistent with previous studies, negative selection was predominant over positive selection [109]. However, no major differences were observed between viruses from farms and the wild. A limitation of the analysis is that the dataset focused on partial NS1 sequence. In the future, it would be of interest to look at longer sequence regions and more thoroughly analyze mutation locations and their effect on protein function.

Table 6. *Positively and negatively selected sites between amino acids 145-244 of NS1 of AMDV strains in Poland. Modified from Table 4 in online supplementary material of Study IV.*

Site of NS1	149	157	159	160	172	175	181	183	188	193	194	207	209	210	211	213	214	217	218	223	234	238	239
All farmed mink			+		−		−	−				+	+	+	−	−	+		−	−			
All feral mink	+	−	+	−		−	−		−	−	−	+	+		−	−	+	−			+	−	−

4.3 VIRUS–HOST DYNAMICS IN WILD MINK

ELISA data was used to analyze AMDV in feral mink across Poland. AMDV antibodies were detected in mink at all study sites with a total prevalence of 69.6%. Seroprevalence (according to the GLM) varied between regions, being highest in the west (82.6%, 95% CI = 79.9–86.7), second highest in the east (63.7%, 95% CI = 59.7–67.4), and lowest in the north (46.1%, 95% CI = 33.8–58.9, pairwise $p < 0.0001$ in all cases). Males (74.5%, 95% CI = 70.9–77.8) were infected more frequently than females (67.9%, CI 95% = 63.1–72.3, $p = 0.0216$), which contradicts previous studies [81-83, 98, 102] but is logical considering that males have larger home ranges than females and travel longer distances, leading to more contacts between individuals. A statistically significant difference that was not detected in other studies may be partly

explained by a larger sample size. Adults were infected more frequently than subadults ($p < 0.0001$), and seroprevalence was higher in the breeding season compared to the non-breeding season ($p < 0.001$); both of these findings can also be explained by a higher number of contacts between individuals (Figure 2 in Study III).

The probability of AMDV infection (according to the GAM) increased when the number of farmed mink in the region increased ($p < 0.001$, Figure 3 in Study III), similar to the results from Canada, Iceland, and Spain [70, 81, 102]. Seroprevalence fluctuated during the study period and peaked every 3–4 years in the region of low farming intensity (NNP and BNP) but remained more constant in the region of high farming intensity (WMNP; Figure 5 in Study III), suggesting a constant flow of virus from farms into the wild. Seropositive mink had worse body conditions than seronegative mink ($p = 0.005$), and their spleens ($p < 0.001$), livers ($p < 0.001$), and kidneys ($p < 0.001$) were also relatively larger (Figures 6 and 7 in Study III). In earlier studies on wild mink, Person et al. also detected higher spleen weights in AMDV-infected individuals but not higher liver or kidney weights, which may also be partly explained by a smaller sample size [99]. Panicz et al. also detected a deterioration in body and spleen condition soon after these results were published [102]. As most studies on AMDV symptoms and their effect on mink fitness have focused on farmed mink or experimental infections, information on the effect of AMDV infection on health, survival, and the population size and density of wild mink is more limited. However, these results indicate a negative effect of AMDV infection on the body condition of wild mink.

4.4 COMPARISON OF DIFFERENT HOST SPECIES

4.4.1 BLUE FOXES (II)

AMDV DNA was detected in 4/29 tested stool samples of farmed blue foxes. In phylogenetic analysis, all strains represented AMDV and were grouped closely with other Finnish strains. On one occasion, we had sequence data from mink and foxes from the same farm (mink 48 and foxes 50–52, Figure 2 in Study II). Strains from mink 48 and foxes 50 and 51 were 99.3% identical based on the pan-AMDV region, and strains from mink 48 and fox 52 were 100% identical based on the pan-AMDO-region, indicating that the foxes had the same virus strains as mink on the farm. AMDV antibodies were found from red fox earlier [87] However, to our knowledge, this was the first report on AMDV DNA in foxes and was later followed by the publication of DNA findings from red foxes in Canada [20]. These results could indicate that foxes can be infected with the same strains as mink and even secrete it in their stool. However, determining whether such transmission is possible would require further studies and confirmation, as contamination during sampling or virus originating from contaminated food cannot be excluded.

4.4.2 AMDV IN NATIVE MUSTELIDS FROM EASTERN POLAND IN COMPARISON TO AMERICAN MINK (IV)

AMDV prevalence in American mink and native mustelids in eastern Poland was compared (Table 7). Seroprevalence was higher in American mink (64%) than in pine martens (35%, p = 3.88E−5) and otters (22%, p = 0.026). It was also higher in stone martens (62%) than in pine martens (p = 0.0038) or otters (p = 0.040; Figure 2 in Study IV). One of the reasons behind the varying seroprevalence between species probably relates to their different living habitats. Stone martens, which have high seroprevalence, inhabit rural areas, disperse far, and can easily enter farms and be exposed to the virus [197]. However, pine martens prefer forests and avoid rural areas and hence have less contact with farmed mink, whereas polecats inhabit rural areas and river and wetland habitats [197, 198]. Contact between mink and otters, which have the lowest seroprevalence, is probably more limited. However, the small sample size prevents strong conclusions.

PCR was used to test the virus presence in seropositive individuals. AMDV DNA was detected in 96% of tested seropositive American mink but only up to 18% of other seropositive mustelids (Table 7). Either the virus strains are too diverse or viral loads too low for PCR to detect, or seropositive, PCR-negative mustelids have cleared the virus. The latter scenario is supported by Canuti et al., who detected similar percentages of PCR-positive martens in Canada and suggested that mink is the maintenance host for AMDV [84]. This result is also supported by the antibody results, which appear to reflect the amount of contact the species probably have with mink. All the sequences from stone martens, pine martens, and polecats represented AMDV, and no new species were found, although pan-AMDO-PCR has been designed to also detect other related species.

Table 7. *Prevalence of ELISA-positive and PCR-positive individuals of wild mustelid species in eastern Poland. Results are reported as percentages, 95% confidence intervals are shown in brackets, and absolute numbers (positive samples/all samples) are shown in parentheses. ELISA results are according to the GLM, and PCR results are reported as percentages of positive individuals from all samples and ELISA-positive samples. Modified from Study IV.*

Species	ELISA	PCR (% of all individuals)	PCR (% of ELISA-positive individuals)
N. vison	64 [60–68] (396/637)	NA	96 [87–100] (52/54*)
M. putorius	48 [26–69] (9/19)	5 [0–14] (1/19)	11 [0–29] (1/9)
M. martes	35 [24–48] (27/63)	6 [0–12] (4/63)	15 [1–28] (4/27)
M. foina	62 [49–74] (40/61)	11 [4–21] (7/61)	18 [6–30] (7/40)
L. lutra	22 [5–58] (2/9)	0 (0/9)	0 (0/2)

* Only a subset of randomly selected ELISA-positive feral mink was included in PCR analysis.

4.5 DISEASE TOLERANCE IN FARMED MINK

4.5.1 ANTIBODY AND PCR RESULTS (V)

Mink from Farm 1 were sampled on four occasions over 2.5 years to follow their IgG and DNA prevalence and health status (Figure 9). A veterinarian judged the mink alive at the end of the follow-up as clinically healthy in all samplings, excluding one mink with signs of clinical AD in the last sampling. The average litter sizes of the mink were comparable to the average litter sizes in Finland (5.0 [SD = 7.39] in 2017, 5.0 [SD = 7.57] in 2018, and 4.0 [SD = 7.32] in 2019). Although all mink were PCR-positive at some point during the follow-up, the seroprevalence only reached 71.4%, and 29%–41% of ELISA-negative mink were PCR-positive. These numbers are similar (40%, 4/10) to earlier observations from another farm with decades of breeding of tolerant mink [199] and somewhat higher than those observed in other studies, where the numbers reached 16.5% [77, 99, 154]. A detectable antibody response can form later than detectable viremia [127]. However, fresh infections do not explain the high proportion of seronegative but PCR-positive mink in this case, as many of the mink remained like this for over two years. The high proportion of persistently infected antibody-negative, PCR-positive mink is probably due to decades of breeding that led to the selection of low antibody producers, as also suggested by Farid et al. [154, 199]. However, as false negative antibody results would explain the unsuccessful eradication attempts on numerous mink farms, further research on freshly infected farms would be required to determine the extent of this phenomenon in a naïve mink population.

No statistically significant difference was found between IgG levels of mink from Farms 1 and 2 ($p = 0.259$). In particular, mink from Farm 1 had high variation in A450, ranging from consistently low/negative to values similar to the mink with clinical AD. The mink with high A450 didn't develop a clinical AD during the follow-up. This is in line with previous observations that even asymptomatic mink can occasionally have high antibody levels and brings up a question about suitability of using just antibody levels in selection for tolerance. A statistically significant difference between color types was only observed between white and brown mink in a 2018 sampling ($p = 0.027$; Figure 1 in Study V). No correlation was observed between A450 and litter size. The mean AMDV genome copy number was higher on Farm 2 than Farm 1 (spleen: $p = 0.006$, kidney: $p = 0.011$). However, no statistically significant difference was found between spleen and kidney (Farm 1: $p = 0.352$, Farm 2: $p = 0.686$) or between color types on Farm 1 ($p = 0.609$; Figure 10, see Table 2 in Study V for exact values). The AMDV genome copy number in the spleen (Spearman's rho = 0.568, $p = 0.027$) and spleen weight as a proportion of body weight (Spearman's rho = 0.589, $p = 0.013$) both positively correlated with A450. However, no statistically significant correlations were observed between other tested factors (Table 3, Study V). It is also noteworthy that the sample sizes are relatively small and can only be used to detect major differences. Detecting minor differences would require a larger sample size.

Sequences from nt 578–951 were used to further inspect virus strains on the farms (Figure 5 in Study V). From Farm 1, 9/9 pan-AMDV sequences from blood samples in March 2017, 9/25 on October 2017, 6/17 on October 2018, and 8/17 on November 2019 were usable, whereas the rest were excluded from the data due to unclear sequences, which were probably caused by coinfection. Additionally, 11 AMDV sequences from spleen samples and three from kidney samples were acquired. Sequences from the spleens of all five mink of Farm 2 were usable. Sequences from Farm 1 were in four separate clusters (tMRCA = 21.2 years, 95% HDP = 9.08–35.34) and sequences from Farm 2 in one cluster (tMRCA = 4.71 years, 95% HDP = 3.19–7.15 years) in the phylogenetic tree. Virus strains in blood changed in at least nine of the mink from Farm 1 during the follow-up, and, in the 2019 sampling, at least four of the mink had different virus strains (2–13 nt difference) in tissues (kidney/spleen) and blood. These mink may have been infected by more than one virus strain, and different strains were dominant in PCRs performed for different tissues, or the virus started evolving differently in different tissues. Because of the frequent coinfection due to several circulating virus strains on Farm 1, no further sequence analysis was performed.

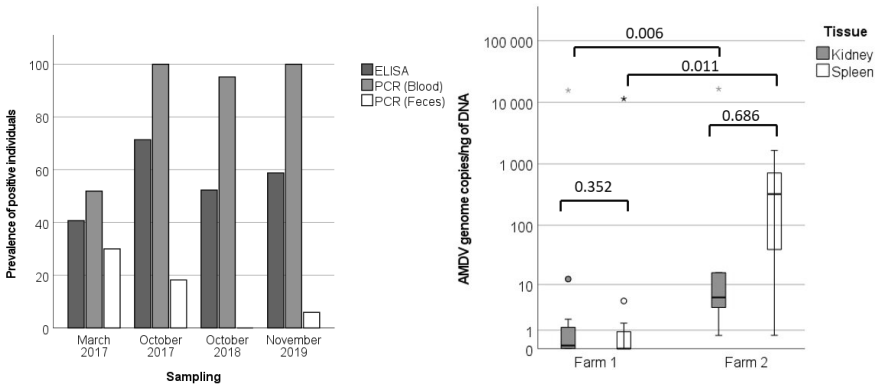

Figure 9 Prevalence (%) of AMDV-positive individuals in ELISA and PCR on Farm 1 during the follow-up (A) and a comparison of AMDV genome copy numbers between Farms 1 and 2 and between tissues together with p-values (B). Differences between tissues were tested with the Wilcoxon signed-rank test and between farms with the independent-samples Mann-Whitney U-test. More detailed test statistics are included in Supplementary Table 2 of Project V.

4.5.2 PATHOLOGY AND HISTOLOGY (V)

In autopsy, only one of the sapphire mink from farm 1 had signs of AD despite all of them being PCR-positive. Others were considered free of AD signs by a veterinarian. In histology, the most severe lesions were observed in the spleen, followed by the liver and lungs, kidney and intestines, and brain. Clinically sick mink had moderate or severe spleen, kidney, brain, and liver lesions, and some of the apparently healthy mink also had mild to moderate spleen, kidney, liver, lung, and intestinal lesions (Table S3 in Study V and Figure 10). Due to the AMDV-negative control mink also having mild/moderate spleen, intestinal, and liver lesions, kidney lesions were considered most specific for AMDV and lesions in other tissues potentially unrelated to AD. Many macroscopically healthy mink had histopathological signs of AD, indicating that they were not completely free from tissue damage, but the disease was either mild or progressing slowly. The only mink with severe clinical AD had also been apparently healthy in the previous samplings of the 2.5-year follow-up. A change in the virus strain to a more pathogenic one is possible. However, as the same virus strain was detected in many asymptomatic mink, host factors, such as those associated with color type, other microbes, and old age, are the most likely factors in the sudden onset of severe AD after a long asymptomatic period. Some of the other mink may have also developed AD had the follow-up been continued. No connections were observed between virus copy numbers and spleen and kidney lesions (Figure S5 in Study V), and the only statistically significant connection between the ELISA level and tissue lesions was observed in the intestines (Figure S7 in Study V). However, the relevance of this observation remains an open question.

To summarize, Farm 1 had litter sizes and pelt quality comparable to the averages in Finland. Mink were often low-antibody producers, had low numbers of virus in their tissues, and had mainly mild histopathological signs of AD at the end of the 2.5-year follow-up. These findings show that it is possible to coexist with the virus if eradication attempts are unsuccessful. Especially in situations where neighboring farms are infected, permanently eradicating the virus from a single farm may be impossible, and other options should be considered. Thus far, the selection of mink has mainly been based on phenotypic health, litter size, pelt quality, IAT, and antibody titers, but more detailed information on the traits used for selection is required to accelerate the process of acquiring a tolerant herd and avoid causing additional health issues with overbreeding.

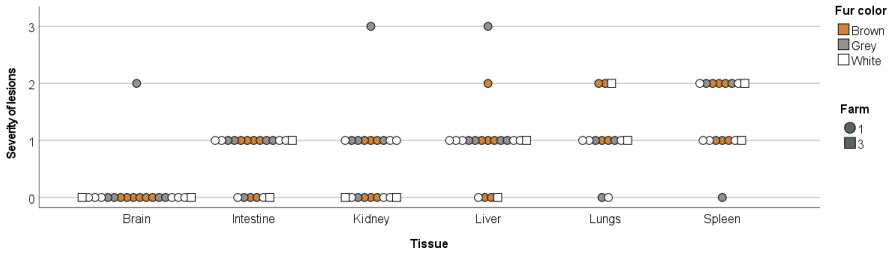

Figure 10 Severity of histopathological lesions in different tissues in AMDV-positive farm (1) and AMDV-negative farm (3).

4.5.3 TRANSCRIPTOME COMPARISON BETWEEN ASYMPTOMATIC, SYMPTOMATIC, AND AMDV-NEGATIVE MINK (V)

Differences in gene expression between farms (three mink/farm) were assessed with transcriptome analysis to learn more about gene-level differences between mink with different disease status (Table 8). All the mink in the transcription analysis were white females, and the samples were collected within a 2-month interval in the late autumn to minimize the differences caused by factors other than AMDV. When comparing infected (Farms 1 and 2) and uninfected mink (Farm 3), fewer upregulated and more downregulated genes were detected on Farm 1 (63 upregulated and 136 downregulated) whereas the opposite situation was observed on Farm 2 (237 upregulated and 184 downregulated). Genes that were highly upregulated ($log_2FC>4$) on Farm 2 compared to the other farms were involved in differentiation, immune response (especially innate immunity), transcription regulation, and apoptosis and highly downregulated genes included several genes involved in trancription regulation, immune response, and differentiation.

Regarding other genes with smaller but significant differential expression on Farm 2, several immune response genes were either upregulated or downregulated on Farm 2 but not on Farm 1. This included upregulation of some proinflammatory cytokines (e.g., IL-1β) but also regulation of several genes, leading to suppression of inflammation, B cell proliferation and antibody production but induction of T cell differentiation (e.g., IL-27, IL12- and IL2-receptors, IL1RL1, and IL1R5A). As Farm 2 reported that the number of mink with symptomatic infection was slowly decreasing, it appears that Farm 2 was also approaching a situation where mink live with the virus and are mainly asymptomatic. This makes these results consistent with the suggestion by Bloom et al. [200] concerning the predominance of the Th1 response (macrophage activation) over the Th2 response (B cell activation and antibody production) in mink lacking the progressive disease and the observation that immunosuppressive medication leads to milder disease [146]. As AMDV uses the host immune response

and antibodies to enter the cells, suppression of inflammation and the immune response (especially antibody response) would be a logical step in virus tolerance. Even though possible coinfections by other microbes affecting the results can't be ruled out, this analysis supports earlier studies and adds new information about gene-level cellular responses during AMDV infection to help plan further studies about disease control and treatment.

Table 8. *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 ($Log_2FC < -4$ or >4) are listed separately.*

	Upregulated genes		**Downregulated genes**	
	N	High difference genes	N	High difference genes
Farm 1 vs Farm 3	63	LMCD1, PRUNE2	136	COL22A1
Farm 2 vs Farm 3	237	G0S2, ELMOD1, GATA6, CD160, GALNT13, LGSN, ONECUT2, LRRC7, OSM, GLI2, NAMPT, COL19A1, NUGGC, BATF2, DDIT4, ARNT2, SCN5A, WSCD2, CCDC178, CAPN14, GZMA	184	ACP7, LEXM, TFPI2, CFAP43, FBXL2, ADCYAP1R1, MAPK4, LYPD1, TUB, EDIL3, SORCS1, APCDD1, NXF5
Farm 2 vs Farm 1	294	PLAUR, DCLK1, NUGGC, CATSPERB, SCN5A, S100A12, NOL3, CCDC178, POU2F3, NR4A2, LGSN, LRRC18, COL4A1, NAMPT, FOS, MYO16, CSTA, OSM, COL22A1, LCN2, PGLYRP1, CD177, GALNT13, ELMOD1, G0S2, ONECUT2	237	NXPH1, FCER1A, COL28A1, CDH5, MAPK4, TUB, LYPD1, ADCYAP1R1, PALD1, EDIL3, OCLN, FBXL2, AASS, PCDH7, HS6ST2, HPGD, SORBS2

5 CONCLUSIONS

This thesis focused on AMDV epidemiology, evolution, and transmission in and between farms and the wild, compared virus–host dynamics in different species, developed and validated PCR and sequencing methods, and studied host factors behind disease severity. The main findings were as follows:

- Probe-based NS1-probe-PCR that was developed during the study had comparable sensitivity and good specificity with diagnostic samples compared to two former SYBR green-based PCRs and was adopted for use in diagnostics to avoid time-consuming Sanger sequencing. Generally, none of the three PCRs can be judged as superior to the others, and the best PCR should be selected based on the intended use.
- Whole AMDV genome sequencing with PCR proved challenging due to the lack of sequence data from genome ends for efficient primer design. A PCR-free sequencing method based on metagenomic protocols is more efficient in amplifying diverse virus strains and other amdoparvoviruses, but further testing and development of sensitivity are probably still required.
- AMDV has arrived in Finland and Poland in several separate events over the years and has been transmitted frequently between countries, although country-specific clustering was also detected.
- In Finland, transmission between farms and the wild several years ago was observed, although due to the uncertainty of molecular clock estimates and sampling biases, recent transmission cannot be excluded. In Poland, ELISA results indicated transmission between farms and the wild. In sequencing, wild and farm strains in Poland were in separate branches.
- Data from wild mustelids in Poland showed that intensive mink farming affected AMDV dynamics in the wild. Sequencing results showed that farming affects AMDV diversity and transmission, although many of the details concerning the transmission mechanisms can only be speculated. ELISA results showed that farming also affects the temporal dynamics and seroprevalence of AMDV in the wild.
- Frequent recombination, coinfection, and a rapid substitution rate were observed.
- Comparison of AMDV prevalence in American mink and native mustelids in Poland indicated that although native mustelids are also frequently infected, the virus does not persist in them as it does in mink.
- Follow-up of mink farm aiming to breed a disease-tolerant mink herd showed that mink can live with the virus for a long time, appear healthy, and have normal litter sizes. These findings indicate the potential of living with AMDV in cases where eradication attempts are unsuccessful and give new insight for further studies on disease control.

6 ACKNOWLEDGMENTS

This work was conducted within the doctoral program of Clinical Veterinary Medicine in the Department of Veterinary Biosciences of the Faculty of Veterinary Medicine and Department of Virology of the Faculty of Medicine, University of Helsinki, Finland. Studies III and IV were conducted in collaboration with the Mammal Research Institute, Polish Academy of Sciences.

I want to express my gratitude to my supervisors, Tarja Sironen, Olli Vapalahti, and Ravi Kant, for their help and guidance. Tarja, whom I have worked with ever since I started in this research unit, has been one of my main advisors even before I began the work for this thesis and has provided me with excellent tools that I can continue to use in my career. Olli is an excellent source of ideas and has provided valuable input for the projects. Ravi has been a great help with the practicalities and writing process and has been the main person in helping with the transcriptome study.

I would also like to thank all my other colleagues from this group who have helped me on the way. This has been a wonderful working environment, the type one can only dream of. Kirsi Aaltonen was an unofficial mentor from the beginning, providing much valuable advice with the protocols, research planning, and troubleshooting. Thank you also for driving with me to collect samples from Ostrobothnia, where we began the day by pushing the car out from a pile of ice and snow and finished it by driving hundreds of kilometers in an awful snowstorm with a car full of smelly samples. Teemu Smura provided much help and advice with the phylogenetics and taught me more about sequence analysis than any bioinformatics course. I also thank our amazing laboratory technicians, Johanna Martikainen, Irina Suomalainen, Mira Utriainen, and Sanna Mäki, for maintaining the reagents and other necessities in the laboratory and helping me whenever I could not find something and Johanna also for extracting DNA from the diagnostic samples used in this thesis. Thank you to Anna Knuuttila for all the preliminary work involved in establishing diagnostic protocols and acquiring the wild mustelid samples from Finland.

I also thank Anna-Maria Moisander-Jylhä for collecting all the samples from Farm 1 and hosting us in Ostrobothnia during the sample collections. Heli Nordgrens' help and expertise in pathology were invaluable. Thank you to Lars Paulin for the transcriptome sequencing. I also thank Paula Kinnunen for integrating me into this research group as a summer student all those years ago and supervising my master's thesis. My master's thesis was not the easiest and included much troubleshooting and frustration. However, it equipped me with valuable critical thinking skills and the ability to conduct independent research.

Regarding other collaborations, I want to thank our Polish collaborators, especially Andrzej Zalewski, for organizing, acquiring, and handling the samples, analyzing the ELISA results, and writing. Thank you also to the other Polish coauthors, Marcin Brzeziński, Marta Kołodziej-Sobocińska, and Władysław Jankow, and technical assistants E. Bujko, D. Chilecki, E. Hapunik, and D. Ławreszuk. Thank you to Jussi

Peura and Majvor Eerola from Fin FurLab for all the information concerning the Finnish fur industry.

I wish to thank everyone involved in sample collections. Diagnostic samples were provided by Fin Furlab. Wild mustelids from Finland and Estonia were from the thesis project of Anna Knuuttila. I also want to thank the farmers who participated in Study V and enabled us to obtain the samples. Thanks also to Sofie Svenns for collecting samples from Farm 2. Sample collection from Poland was managed by our collaborators.

The studies conducted in Finland were funded by the Finnish Fur Breeders' Association, the Finnish Veterinary Foundation, and the Finnish Veterinary Association. Sample collection and analysis conducted in Poland was funded by the National Science Centre, Poland fund acquired by our collaborators.

I wish to thank the thesis committee members Jussi Peura and Kati Hokynar for helpful discussion in the committee meetings, pre-examiners Petri Susi and Per Saris for their critical review and feedback, and Scribbr for revising the language of this thesis. I am also grateful to Marta Canuti for agreeing to be an opponent for this work.

Finally, I want to thank my family, relatives, and friends. Special thanks go to my parents and sister. My interest in biology began in childhood when we spent a lot of time exploring the nature, did "scientific" experiments with whatever we could find from home, and played with a toy microscope. Thank you and apologies also for the countless of discussions about microbiology-related topics like disgusting sample materials and gut microbiome in the dinner table. Mira, my friend, thank you for the years of friendship and all the fun free-time activities you always come up with. Eemeli, thank you for the love and support and sharing my passion for nature. All the hiking trips exploring the Finnish nature out of email connections and stress are truly invaluable. Please, keep up encouraging me to explore new places and try new things.

7 REFERENCES

1. Bonesi, L. and S. Palazon, *The American mink in Europe: Status, impacts, and control.* Biological conservation, 2007. **134**: p. 470 – 483.
2. Nummi, P., *Suomeen istutetut riistaelaimet*. 1988: Helsingin yliopisto, Maatalous- ja Metsäeläintieteen Laitos.
3. Brzezinski, M., et al., *Expansion and population dynamics of a non-native invasive species: the 40-year history of American mink colonisation of Poland.* Biol Invasions, 2019. **21**: p. 531–545.
4. Zalewski, A., et al., *High mitochondrial DNA diversity of an introduced alien carnivore: comparison of feral and ranch American mink Neovison vison in Poland.* Diversity and Distributions, 2011. **17**: p. 757–768.
5. Gorham, J.R., et al., *The epizootiology of aleutian disease.* Front Biol, 1976. **44**: p. 135-58.
6. Knuuttila, A., *Diagnostics and epidemiology of Aleutian mink disease virus*, in *Faculty of Veterinary Medicine and Faculty of Medicine*. 2015, University of Helsinki: *Dissertationes Schola Doctoralis Sceintiae Circumiectalis, Alimentariae, Biologicae, Universitatis Helsinkiensis*.
7. FIFUR. *Finnish fur breeders' association web page*. 2021 [cited 2021 20.8]; Available from: https://fifur.fi/.
8. Rabalski, L., et al., *Severe acute respiratory syndrome coronavirus 2 in farmed mink (Neovison vison), Poland.* Emerg Infect Dis, 2021. **27**(9): p. 2333-2339.
9. ICTVdb. 2021 [cited 2021; Available from: https://talk.ictvonline.org/.
10. Penzes, J.J., et al., *Reorganizing the family Parvoviridae: a revised taxonomy independent of the canonical approach based on host association.* Arch Virol, 2020. **165**(9): p. 2133-2146.
11. Cotmore, S.F., et al., *ICTV Virus Taxonomy Profile: Parvoviridae.* J Gen Virol, 2019. **100**(3): p. 367-368.
12. Jager, M.C., et al., *Small but mighty: old and new parvoviruses of veterinary significance.* Virol J, 2021. **18**(1): p. 210.
13. Chesebro, B., et al., *Purification and ultrastructure of Aleutian disease virus of mink.* Nature, 1975. **254**(5499): p. 456-7.
14. Cho, H.J., *Purification and structure of aleutian disease virus.* Front Biol, 1976. **44**: p. 159-74.
15. Cho, H.J. and D.G. Ingram, *The antigen and virus of Aleutian disease in mink.* J Immunol Methods, 1974. **4**(2): p. 217-28.
16. Li, L., et al., *Novel amdovirus in gray foxes.* Emerg Infect Dis, 2011. **17**(10): p. 1876-8.
17. Shao, X.Q., et al., *Novel amdoparvovirus infecting farmed raccoon dogs and arctic foxes.* Emerg Infect Dis, 2014. **20**(12): p. 2085-8.
18. Canuti, M., et al., *Full genetic characterization and epidemiology of a novel amdoparvovirus in striped skunk (Mephitis mephitis).* Emerg Microbes Infect, 2017. **6**(5): p. e30.
19. Alex, C.E., et al., *Amdoparvovirus infection in red pandas (Ailurus fulgens).* Vet Pathol, 2018. **55**(4): p. 552-561.
20. Canuti, M., et al., *Multi-host dispersal of known and novel carnivore amdoparvoviruses.* Virus Evol, 2020. **6**(2).
21. Bodewes, R., et al., *Viral metagenomic analysis of feces of wild small carnivores.* Virol J, 2014. **11**: p. 89.

22. Yang, S., et al., *Viral metagenomics reveals diverse viruses in the feces samples of raccoon dogs.* Front Vet Sci, 2021. **8**: p. 693564.
23. Nituch, L.A., et al., *Aleutian mink disease virus in striped skunks (Mephitis mephitis): evidence for cross-species spillover.* J Wildl Dis, 2015. **51**(2): p. 389-400.
24. Shahrabadi, M.S., H.J. Cho, and R.G. Marusyk, *Characterization of the protein and nucleic acid of Aleutian disease virus.* J Virol, 1977. **23**(2): p. 353-62.
25. Bloom, M.E., R.E. Race, and J.B. Wolfinbarger, *Characterization of Aleutian disease virus as a parvovirus.* J Virol, 1980. **35**(3): p. 836-43.
26. Bloom, M.E., et al., *Nucleotide sequence of the 5'-terminal palindrome of Aleutian mink disease parvovirus and construction of an infectious molecular clone.* J Virol, 1990. **64**(7): p. 3551-6.
27. Bloom, M.E., et al., *Nucleotide sequence and genomic organization of Aleutian mink disease parvovirus (ADV): sequence comparisons between a nonpathogenic and a pathogenic strain of ADV.* J Virol, 1988. **62**(8): p. 2903-15.
28. Alexandersen, S., M.E. Bloom, and S. Perryman, *Detailed transcription map of Aleutian mink disease parvovirus.* J Virol, 1988. **62**(10): p. 3684-94.
29. Qiu, J., et al., *The transcription profile of Aleutian mink disease virus in CRFK cells is generated by alternative processing of pre-mRNAs produced from a single promoter.* J Virol, 2006. **80**(2): p. 654-62.
30. Bloom, M.E., R.E. Race, and J.B. Wolfinbarger, *Identification of a nonvirion protein of Aleutian disease virus: mink with Aleutian disease have antibody to both virion and nonvirion proteins.* J Virol, 1982. **43**(2): p. 608-16.
31. Christensen, J., et al., *Purification and characterization of the major nonstructural protein (NS-1) of Aleutian mink disease parvovirus.* J Virol, 1995. **69**(3): p. 1802-9.
32. Huang, Q., et al., *Molecular characterization of the small nonstructural proteins of parvovirus Aleutian mink disease virus (AMDV) during infection.* Virology, 2014. **452-453**: p. 23-31.
33. McKenna, R., et al., *Three-dimensional structure of Aleutian mink disease parvovirus: implications for disease pathogenicity.* J Virol, 1999. **73**(8): p. 6882-91.
34. Willwand, K. and O.R. Kaaden, *Proteins of viral and cellular origin bind to the Aleutian disease virus (ADV) DNA 3'-terminal hairpin: presentation of a scheme for encapsidation of ADV DNA.* J Virol, 1990. **64**(4): p. 1598-605.
35. Bloom, M.E., et al., *Identification of Aleutian mink disease parvovirus capsid sequences mediating antibody-dependent enhancement of infection, virus neutralization, and immune complex formation.* J Virol, 2001. **75**(22): p. 11116-27.
36. Kanno, H., J.B. Wolfinbarger, and M.E. Bloom, *Aleutian mink disease parvovirus infection of mink macrophages and human macrophage cell line U937: demonstration of antibody-dependent enhancement of infection.* J Virol, 1993. **67**(12): p. 7017-24.
37. Bournazos, S., A. Gupta, and J.V. Ravetch, *The role of IgG Fc receptors in antibody-dependent enhancement.* Nat Rev Immunol, 2020. **20**(10): p. 633-643.
38. Dworak, L.J., J.B. Wolfinbarger, and M.E. Bloom, *Aleutian mink disease parvovirus infection of K562 cells is antibody-dependent and is mediated via an Fc(gamma)RII receptor.* Arch Virol, 1997. **142**(2): p. 363-73.
39. Wohlsein, P., et al., *Antigen distribution in organs of mink with Aleutian disease parvovirus infection.* Zentralbl Veterinarmed B, 1990. **37**(9): p. 651-9.
40. Alexandersen, S., M.E. Bloom, and J. Wolfinbarger, *Evidence of restricted viral replication in adult mink infected with Aleutian disease of mink parvovirus.* J Virol, 1988. **62**(5): p. 1495-507.
41. Mori, S., et al., *Replication of Aleutian mink disease parvovirus in lymphoid tissues of adult mink: involvement of follicular dendritic cells and macrophages.* J Virol, 1991. **65**(2): p. 952-6.
42. Jensen, K.T., et al., *Replication of Aleutian mink disease parvovirus in mink lymph node histocultures.* J Gen Virol, 2000. **81**(Pt 2): p. 335-43.

43. Bloom, M.E., et al., *Analysis of Aleutian disease virus infection in vitro and in vivo: demonstration of Aleutian disease virus DNA in tissues of infected mink.* J Virol, 1985. **55**(3): p. 696-703.
44. Alexandersen, S., et al., *In situ molecular hybridization for detection of Aleutian mink disease parvovirus DNA by using strand-specific probes: identification of target cells for viral replication in cell cultures and in mink kits with virus-induced interstitial pneumonia.* J Virol, 1987. **61**(8): p. 2407-19.
45. Alexandersen, S., et al., *Passive transfer of antiviral antibodies restricts replication of Aleutian mink disease parvovirus in vivo.* J Virol, 1989. **63**(1): p. 9-17.
46. Alexandersen, S. and M.E. Bloom, *Studies on the sequential development of acute interstitial pneumonia caused by Aleutian disease virus in mink kits.* J Virol, 1987. **61**(1): p. 81-6.
47. Markarian, N.M. and L. Abrahamyan, *AMDV Vaccine: Challenges and Perspectives.* Viruses, 2021. **13**(9).
48. Zadori, Z., et al., *A viral phospholipase A2 is required for parvovirus infectivity.* Dev Cell, 2001. **1**(2): p. 291-302.
49. Best, S.M., et al., *Caspase cleavage of the nonstructural protein NS1 mediates replication of Aleutian mink disease parvovirus.* J Virol, 2003. **77**(9): p. 5305-12.
50. Connolly, P.F. and H.O. Fearnhead, *Viral hijacking of host caspases: an emerging category of pathogen-host interactions.* Cell Death Differ, 2017. **24**(8): p. 1401-1410.
51. Cheng, F., et al., *The capsid proteins of Aleutian mink disease virus activate caspases and are specifically cleaved during infection.* J Virol, 2010. **84**(6): p. 2687-96.
52. Porter, D.D., A.E. Larsen, and H.G. Porter, *Aleutian disease of mink.* Adv Immunol, 1980. **29**: p. 261-86.
53. Jensen, T.H., A.S. Hammer, and M. Chriel, *Monitoring chronic infection with a field strain of Aleutian mink disease virus.* Vet Microbiol, 2014. **168**(2-4): p. 420-7.
54. Padgett, G.A., J.R. Gorham, and J.B. Henson, *Epizootiologic studies of Aleutian disease. I. Transplacental transmission of the virus.* J Infect Dis, 1967. **117**(1): p. 35-8.
55. Broll, S. and S. Alexandersen, *Investigation of the pathogenesis of transplacental transmission of Aleutian mink disease parvovirus in experimentally infected mink.* J Virol, 1996. **70**(3): p. 1455-66.
56. Farid, A.H., P.M. Daftarian, and J. Fatehi, *Transmission dynamics of Aleutian mink disease virus on a farm under test and removal scheme.* J Vet Sci Med Diagn, 2018. **7**(2).
57. Hahn, E.C., L. Ramos, and A.J. Kenyon, *Properties of Aleutian disease virus assayed with feline kidney cells.* Arch Virol, 1977. **55**(4): p. 315-26.
58. Hussain, I., G.W. Price, and A.H. Farid, *Inactivation of Aleutian mink disease virus through high temperature exposure in vitro and under field-based composting conditions.* Vet Microbiol, 2014. **173**(1-2): p. 50-8.
59. Eterpi, M., G. McDonnell, and V. Thomas, *Disinfection efficacy against parvoviruses compared with reference viruses.* J Hosp Infect, 2009. **73**(1): p. 64-70.
60. Prieto, A., et al., *Distribution of Aleutian mink disease virus contamination in the environment of infected mink farms.* Vet Microbiol, 2017. **204**: p. 59-63.
61. Prieto, A., et al., *Application of real-time PCR to detect Aleutian Mink Disease Virus on environmental farm sources.* Vet Microbiol, 2014. **173**(3-4): p. 355-9.
62. Diaz Cao, J.M., et al., *Molecular assessment of visitor personal protective equipment contamination with the Aleutian mink disease virus and porcine circovirus-2 in mink and porcine farms.* PLoS One, 2018. **13**(8): p. e0203144.
63. Christensen, L.S., et al., *Diversity and stability of Aleutian mink disease virus during bottleneck transitions resulting from eradication in domestic mink in Denmark.* Vet Microbiol, 2011. **149**(1-2): p. 64-71.

64. Farid, A.H., et al., *Prevalence of the Aleutian mink disease virus infection in Nova Scotia, Canada.* Prev Vet Med, 2012. **106**(3-4): p. 332-8.
65. Espregueira Themudo, G., et al., *Identification of biosecurity measures and spatial variables as potential risk factors for Aleutian disease in Danish mink farms.* Prev Vet Med, 2012. **107**(1-2): p. 134-41.
66. Prieto, A., et al., *Lesser housefly (Fannia canicularis) as possible mechanical vector for Aleutian mink disease virus.* Vet Microbiol, 2018. **221**: p. 90-93.
67. Shen, D.T., et al., *The persistence of Aleutian disease virus in the mosquito Aedes fitchii.* Arch Gesamte Virusforsch, 1973. **40**(3): p. 375-81.
68. Yang, Z., et al., *Virome analysis of ticks in a forest region of Liaoning, China: characterization of a novel hepe-like virus sequence.* Virol J, 2021. **18**(1): p. 163.
69. Zaleska-Wawro, M., et al., *Seroprevalence and molecular epidemiology of Aleutian disease in various countries during 1972-2021: A review and meta-analysis.* Animals (Basel), 2021. **11**(10).
70. Nituch, L.A., et al., *Mink farms predict Aleutian disease exposure in wild American mink.* PLoS One, 2011. **6**(7): p. e21693.
71. Knuuttila, A., et al., *Aleutian mink disease virus in free-ranging mustelids in Finland - a cross-sectional epidemiological and phylogenetic study.* J Gen Virol, 2015. **96**(Pt 6): p. 1423-1435.
72. Nituch, L.A., et al., *Molecular epidemiology of Aleutian disease virus in free-ranging domestic, hybrid, and wild mink.* Evol Appl, 2012. **5**(4): p. 330-40.
73. Jakubczak, A., et al., *Comparative molecular analysis of strains of the Aleutian Disease Virus isolated from farmed and wild mink.* Ann Agric Environ Med, 2017. **24**(3): p. 366-371.
74. Leimann, A., et al., *Molecular epidemiology of Aleutian mink disease virus (AMDV) in Estonia, and a global phylogeny of AMDV.* Virus Res, 2015. **199**: p. 56-61.
75. Ryt-Hansen, P., et al., *Global phylogenetic analysis of contemporary aleutian mink disease viruses (AMDVs).* Virol J, 2017. **14**(1): p. 231.
76. Prieto, A., et al., *Molecular epidemiology of Aleutian mink disease virus causing outbreaks in mink farms from Southwestern Europe: a retrospective study from 2012 to 2019.* J Vet Sci, 2020. **21**(4): p. e65.
77. Wang, Z., et al., *Molecular epidemiology of Aleutian mink disease virus in China.* Virus Res, 2014. **184**: p. 14-9.
78. Kowalczyk, M., B. Horecka, and A. Jakubczak, *Aleutian mink disease virus in the breeding environment in Poland and its place in the global epidemiology of AMDV.* Virus Res, 2019. **270**: p. 197665.
79. Franzo, G., et al., *Impact of viral features, host jumps and phylogeography on the rapid evolution of Aleutian mink disease virus (AMDV).* Sci Rep, 2021. **11**(1): p. 16464.
80. Manas, S., et al., *Aleutian mink disease parvovirus in wild riparian carnivores in Spain.* J Wildl Dis, 2001. **37**(1): p. 138-44.
81. Manas, S., et al., *Prevalence of antibody to Aleutian mink disease virus in European Mink (Mustela Lutreola) and American Mink (Neovison Vison) in Spain.* J Wildl Dis, 2016. **52**(1): p. 22-32.
82. Farid, A.H., *Aleutian mink disease virus in furbearing mammals in Nova Scotia, Canada.* Acta Vet Scand, 2013. **55**: p. 10.
83. Fournier-Chambrillon, C., et al., *Antibodies to Aleutian mink disease parvovirus in free-ranging European mink (Mustela lutreola) and other small carnivores from southwestern France.* J Wildl Dis, 2004. **40**(3): p. 394-402.
84. Canuti, M., et al., *Ecology and infection dynamics of multi-host Amdoparvoviral and Protoparvoviral carnivore pathogens.* Pathogens, 2020. **9**(2).
85. Porter, H.G., D.D. Porter, and A.E. Larsen, *Aleutian disease in ferrets.* Infect Immun, 1982. **36**(1): p. 379-86.

86. Glueckert, E., et al., *Endemic Skunk amdoparvovirus in free-ranging striped skunks (Mephitis mephitis) in California.* Transbound Emerg Dis, 2019. **66**(6): p. 2252-2263.
87. Ingram, D.G. and H.J. Cho, *Aleutian disease in mink: virology, immunology and pathogenesis.* J Rheumatol, 1974. **1**(1): p. 74-92.
88. LaDouceur, E.E., et al., *Aleutian disease: An emerging disease in free-ranging striped skunks (Mephitis mephitis) from California.* Vet Pathol, 2015. **52**(6): p. 1250-3.
89. Oie, K.L., et al., *The relationship between capsid protein (VP2) sequence and pathogenicity of Aleutian mink disease parvovirus (ADV): a possible role for raccoons in the transmission of ADV infections.* J Virol, 1996. **70**(2): p. 852-61.
90. Britton, A.P., et al., *Beyond rabies: Are free-ranging skunks (Mephitis mephitis) in British Columbia reservoirs of emerging infection?* Transbound Emerg Dis, 2017. **64**(2): p. 603-612.
91. Giannitti, F., et al., *Aleutian disease virus-like virus (Amdoparvovirus Sp.) infecting free-ranging striped skunks (Mephitis Mephitis) in the midwestern USA.* J Wildl Dis, 2018. **54**(1): p. 186-188.
92. Allender, M.C., et al., *Infection with Aleutian disease virus-like virus in a captive striped skunk.* J Am Vet Med Assoc, 2008. **232**(5): p. 742-6.
93. Pennick, K.E., et al., *Aleutian disease in two domestic striped skunks (Mephitis mephitis).* Vet Pathol, 2007. **44**(5): p. 687-90.
94. Kenyon, A.J., B.J. Kenyon, and E.C. Hahn, *Protides of the Mustelidae: immunoresponse of mustelids to Aleutian mink disease virus.* Am J Vet Res, 1978. **39**(6): p. 1011-5.
95. Alexandersen, S., et al., *Experimental transmission of Aleutian disease virus (ADV) to different animal species.* Acta Pathol Microbiol Immunol Scand B, 1985. **93**(3): p. 195-200.
96. McGuire, T.C. and T.B. Crawford, *Antibodies to Aleutian disease virus in human sera.* J Infect Dis, 1980. **142**(4): p. 625.
97. Jepsen, J.R., et al., *Aleutian mink disease virus and humans.* Emerg Infect Dis, 2009. **15**(12): p. 2040-2.
98. Yamaguchi, N. and D.W. Macdonald, *Detection of Aleutian disease antibodies in feral American mink in southern England.* Vet Rec, 2001. **149**(16): p. 485-8.
99. Persson, S., et al., *Aleutian mink disease virus in free-ranging mink from Sweden.* PLoS One, 2015. **10**(3): p. e0122194.
100. Farid, A.H., et al., *A survey of Aleutian mink disease virus infection of feral American mink in Nova Scotia.* Can Vet J, 2010. **51**(1): p. 75-7.
101. Jensen, T.H., et al., *High prevalence of Aleutian mink disease virus in free-ranging mink on a remote Danish island.* J Wildl Dis, 2012. **48**(2): p. 497-502.
102. Panicz, R., et al., *Assessment of Aleutian mink disease virus (AMDV) prevalence in feral American mink in Iceland. Case study of a pending epizootiological concern in Europe.* PeerJ, 2021. **9**: p. e12060.
103. Cho, H.J. and J. Greenfield, *Eradication of Aleutian disease of mink by eliminating positive counterimmunoelectrophoresis test reactors.* J Clin Microbiol, 1978. **7**(1): p. 18-22.
104. Sanchez-Migallon Guzman, D., et al., *Aleutian disease serology, protein electrophoresis, and pathology of the European mink (Mustela lutreola) from Navarra, Spain.* J Zoo Wildl Med, 2008. **39**(3): p. 305-13.
105. Bowman, J., et al., *Testing for Aleutian mink disease virus in the river otter (Lontra canadensis) in sympatry with infected American mink (Neovison vison).* J Wildl Dis, 2014. **50**(3): p. 689-93.
106. Duffy, S., L.A. Shackelton, and E.C. Holmes, *Rates of evolutionary change in viruses: patterns and determinants.* Nat Rev Genet, 2008. **9**(4): p. 267-76.

107. Hoelzer, K., et al., *Phylogenetic analysis reveals the emergence, evolution and dispersal of carnivore parvoviruses.* J Gen Virol, 2008. **89**(Pt 9): p. 2280-2289.
108. Shackelton, L.A., et al., *High rate of viral evolution associated with the emergence of carnivore parvovirus.* Proc Natl Acad Sci U S A, 2005. **102**(2): p. 379-84.
109. Canuti, M., et al., *Driving forces behind the evolution of the Aleutian mink disease parvovirus in the context of intensive farming.* Virus Evol, 2016. **2**(1): p. vew004.
110. Lukashov, V.V. and J. Goudsmit, *Evolutionary relationships among parvoviruses: virus-host coevolution among autonomous primate parvoviruses and links between adeno-associated and avian parvoviruses.* J Virol, 2001. **75**(6): p. 2729-40.
111. Pereira, C.A., E.S. Leal, and E.L. Durigon, *Selective regimen shift and demographic growth increase associated with the emergence of high-fitness variants of canine parvovirus.* Infect Genet Evol, 2007. **7**(3): p. 399-409.
112. Knuuttila, A., et al., *Molecular epidemiology of Aleutian mink disease virus in Finland.* Vet Microbiol, 2009. **133**(3): p. 229-38.
113. Horiuchi, M., et al., *Differences in the evolutionary pattern of feline panleukopenia virus and canine parvovirus.* Virology, 1998. **249**(2): p. 440-52.
114. Shackelton, L.A., et al., *Comparative analysis reveals frequent recombination in the parvoviruses.* J Gen Virol, 2007. **88**(Pt 12): p. 3294-3301.
115. Mochizuki, M., et al., *Recombination between vaccine and field strains of canine parvovirus is revealed by isolation of virus in canine and feline cell cultures.* J Vet Med Sci, 2008. **70**(12): p. 1305-14.
116. Wang, J., et al., *Evidence for natural recombination between mink enteritis virus and canine parvovirus.* Virol J, 2012. **9**: p. 252.
117. Karstad, L. and T.J. Pridham, *Aleutian disease of Mink: I. Evidence of its Viral Etiology.* Can J Comp Med Vet Sci, 1962. **26**(5): p. 97-102.
118. Eklund, C.M., et al., *Aleutian disease of mink: properties of the etiologic agent and the host responses.* J Infect Dis, 1968. **118**(5): p. 510-26.
119. Porter, D.D., A.E. Larsen, and H.G. Porter, *The pathogenesis of Aleutian disease of mink. I. In vivo viral replication and the host antibody response to viral antigen.* J Exp Med, 1969. **130**(3): p. 575-93.
120. Porter, D.D., *Aleutian disease: a persistent parvovirus infection of mink with a maximal but ineffective host humoral immune response.* Prog Med Virol, 1986. **33**: p. 42-60.
121. Alexandersen, S., *Acute interstitial pneumonia in mink kits: experimental reproduction of the disease.* Vet Pathol, 1986. **23**(5): p. 579-88.
122. Aasted, B., *Mink infected with Aleutian disease virus have an elevated level of CD8-positive T-lymphocytes.* Vet Immunol Immunopathol, 1989. **20**(4): p. 375-85.
123. Chen, W. and B. Aasted, *Analyses of leucocytes in blood and lymphoid tissues from mink infected with Aleutian mink disease parvovirus (AMDV).* Vet Immunol Immunopathol, 1998. **63**(4): p. 317-34.
124. Porter, D.D., H.G. Porter, and A.E. Larsen, *Aleutian disease parvovirus infection of mink and ferrets elicits an antibody response to a second nonstructural viral protein.* J Virol, 1990. **64**(4): p. 1859-60.
125. Hadlow, W.J., R.E. Race, and R.C. Kennedy, *Royal pastel mink respond variously to inoculation with Aleutian disease virus of low virulence.* J Virol, 1984. **50**(1): p. 38-41.
126. Porter, D.D., et al., *Immunoglobulin classes of Aleutian disease virus antibody.* Infect Immun, 1984. **43**(2): p. 463-6.
127. Farid, A.H., I. Hussain, and I. Arju, *Detection of Aleutian mink disease virus DNA and antiviral antibodies in American mink (Neovison vison) 10 days postinoculation.* J Vet Diagn Invest, 2015. **27**(3): p. 287-94.
128. Jensen, T.H., M. Chriel, and M.S. Hansen, *Progression of experimental chronic Aleutian mink disease virus infection.* Acta Vet Scand, 2016. **58**(1): p. 35.

129. Bloom, M.E., et al., *Aleutian disease of mink: the antibody response of sapphire and pastel mink to Aleutian disease virus.* J Immunol, 1975. **115**(4): p. 1034-7.
130. Hadlow, W.J., R.E. Race, and R.C. Kennedy, *Temporal replication of the Pullman strain of Aleutian disease virus in royal pastel mink.* J Virol, 1985. **55**(3): p. 853-6.
131. Hadlow, W.J., R.E. Race, and R.C. Kennedy, *Comparative pathogenicity of four strains of Aleutian disease virus for pastel and sapphire mink.* Infect Immun, 1983. **41**(3): p. 1016-23.
132. Kowalczyk, M., et al., *A comparative molecular characterization of AMDV strains isolated from cases of clinical and subclinical infection.* Virus Genes, 2018. **54**(4): p. 561-569.
133. Henson, J.B., et al., *Experimental hypergammaglobulinemia in mink.* J Exp Med, 1962. **116**: p. 357-64.
134. Anistoroaei, R., A.K. Krogh, and K. Christensen, *A frameshift mutation in the LYST gene is responsible for the Aleutian color and the associated Chediak-Higashi syndrome in American mink.* Anim Genet, 2013. **44**(2): p. 178-83.
135. Johnson, M.I., J.B. Henson, and J.R. Gorham, *The influence of genotype on the development of glomerular lesions in mink with Aleutian disease virus.* Am J Pathol, 1975. **81**(2): p. 321-36.
136. Larsen, A.E. and D.D. Porter, *Pathogenesis of Aleutian disease of mink: identification of nonpersistent infections.* Infect Immun, 1975. **11**(1): p. 92-4.
137. Karimi, K., et al., *Detection of selection signatures for response to Aleutian mink disease virus infection in American mink.* Sci Rep, 2021. **11**(1): p. 2944.
138. Jensen, T.H., et al., *Implementation and validation of a sensitive PCR detection method in the eradication campaign against Aleutian mink disease virus.* J Virol Methods, 2011. **171**(1): p. 81-5.
139. Knuuttila, A., et al., *Validation of an automated ELISA system for detection of antibodies to Aleutian mink disease virus using blood samples collected in filter paper strips.* Virol J, 2014. **11**: p. 141.
140. Knuuttila, A., et al., *Development and evaluation of an enzyme-linked immunosorbent assay based on recombinant VP2 capsids for the detection of antibodies to Aleutian mink disease virus.* Clin Vaccine Immunol, 2009. **16**(9): p. 1360-5.
141. Aasted, B., S. Alexandersen, and J. Christensen, *Vaccination with Aleutian mink disease parvovirus (AMDV) capsid proteins enhances disease, while vaccination with the major non-structural AMDV protein causes partial protection from disease.* Vaccine, 1998. **16**(11-12): p. 1158-65.
142. Porter, D.D., A.E. Larsen, and H.G. Porter, *The pathogenesis of Aleutian disease of mink. II. Enhancement of tissue lesions following the administration of a killed virus vaccine or passive antibody.* J Immunol, 1972. **109**(1): p. 1-7.
143. Karstad, L., T.J. Pridham, and D.P. Gray, *Aleutian disease (Plasmacytosis) of mink: II. responses of mink to formalin-treated diseased tissues and to subsequent challenge with virulent inoculum.* Can J Comp Med Vet Sci, 1963. **27**(5): p. 124-8.
144. Castelruiz, Y., M. Blixenkrone-Moller, and B. Aasted, *DNA vaccination with the Aleutian mink disease virus NS1 gene confers partial protection against disease.* Vaccine, 2005. **23**(10): p. 1225-31.
145. Liu, D., et al., *Construction and immunogenicity analysis of whole-gene mutation DNA vaccine of Aleutian mink virus isolated virulent strain.* Viral Immunol, 2018. **31**(1): p. 69-77.
146. Cheema, A., J.B. Henson, and J.R. Gorham, *Aleutian disease of mink. Prevention of lesions by immunosuppression.* Am J Pathol, 1972. **66**(3): p. 543-56.
147. Kowalczyk, M., et al., *Breeding parameters on a mink farm infected with Aleutian mink disease virus following the use of methisoprinol.* Arch Virol, 2019. **164**(11): p. 2691-2698.

148. Lu, T., et al., *Aptamer-targeting of Aleutian mink disease virus (AMDV) can be an effective strategy to inhibit virus replication.* Sci Rep, 2021. **11**(1): p. 4649.
149. Ryt-Hansen, P., et al., *Outbreak tracking of Aleutian mink disease virus (AMDV) using partial NS1 gene sequencing.* Virol J, 2017. **14**(1): p. 119.
150. Zalewski, A., et al., *Aleutian mink disease: Spatio-temporal variation of prevalence and influence on the feral American mink.* Transbound Emerg Dis, 2021. **68**(4): p. 2556-2570.
151. Gong, Q.L., et al., *Mink Aleutian disease seroprevalence in China during 1981-2017: A systematic review and meta-analysis.* Microb Pathog, 2020. **139**: p. 103908.
152. Themudo, G.E., J. Ostergaard, and A.K. Ersboll, *Persistent spatial clusters of plasmacytosis among Danish mink farms.* Prev Vet Med, 2011. **102**(1): p. 75-82.
153. Gunnarsson, E., *Documenting freedom from disease and re-establishing a free status after a breakdown Aleutian disease (plasmacytosis) in farmed mink in Iceland.* Acta Veterinaria Scandinavica, 2001. **42**: p. 87-87.
154. Farid, A.H. and L.E. Ferns, *Reduced severity of histopathological lesions in mink selected for tolerance to Aleutian mink disease virus infection.* Res Vet Sci, 2017. **111**: p. 127-134.
155. Farid, A.H. and I. Hussain, *Dose response of black American mink to Aleutian mink disease virus.* Immun Inflamm Dis, 2020. **8**(2): p. 150-164.
156. Andersson, A.M., A.K. Nyman, and P. Wallgren, *A retrospective cohort study estimating the individual Aleutian disease progress in female mink using a VP2 ELISA and its association to reproductive performance.* Prev Vet Med, 2017. **140**: p. 60-66.
157. Hagberg, E.E., et al., *A fast and robust method for whole genome sequencing of the Aleutian Mink Disease Virus (AMDV) genome.* J Virol Methods, 2016. **234**: p. 43-51.
158. Conceicao-Neto, N., et al., *Modular approach to customise sample preparation procedures for viral metagenomics: a reproducible protocol for virome analysis.* Sci Rep, 2015. **5**: p. 16532.
159. Tamura, K., et al., *MEGA6: Molecular Evolutionary Genetics Analysis version 6.0.* Mol Biol Evol, 2013. **30**(12): p. 2725-9.
160. Okonechnikov, K., et al., *Unipro UGENE: a unified bioinformatics toolkit.* Bioinformatics, 2012. **28**(8): p. 1166-7.
161. Langmead, B. and S.L. Salzberg, *Fast gapped-read alignment with Bowtie 2.* Nat Methods, 2012. **9**(4): p. 357-9.
162. Li, H., *Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM*. 2013, arXiv.
163. Bolger, A.M., M. Lohse, and B. Usadel, *Trimmomatic: a flexible trimmer for Illumina sequence data.* Bioinformatics, 2014. **30**(15): p. 2114-20.
164. Li, D., et al., *MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph.* Bioinformatics, 2015. **31**(10): p. 1674-6.
165. Somervuo, P. and L. Holm, *SANSparallel: interactive homology search against Uniprot.* Nucleic Acids Res, 2015. **43**(W1): p. W24-9.
166. Plyusnin, I., et al., *Novel NGS pipeline for virus discovery from a wide spectrum of hosts and sample types.* Virus Evol, 2020. **6**(2): p. veaa091.
167. Thompson, J.D., D.G. Higgins, and T.J. Gibson, *CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.* Nucleic Acids Res, 1994. **22**(22): p. 4673-80.
168. Edgar, R.C., *MUSCLE: multiple sequence alignment with high accuracy and high throughput.* Nucleic Acids Res, 2004. **32**(5): p. 1792-7.
169. Martin, D. and E. Rybicki, *RDP: detection of recombination amongst aligned sequences.* Bioinformatics, 2000. **16**(6): p. 562-3.

170. Padidam, M., S. Sawyer, and C.M. Fauquet, *Possible emergence of new geminiviruses by frequent recombination.* Virology, 1999. **265**(2): p. 218-25.
171. Salminen, M.O., et al., *Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning.* AIDS Res Hum Retroviruses, 1995. **11**(11): p. 1423-5.
172. Smith, J.M., *Analyzing the mosaic structure of genes.* J Mol Evol, 1992. **34**(2): p. 126-9.
173. Posada, D. and K.A. Crandall, *Evaluation of methods for detecting recombination from DNA sequences: computer simulations.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 13757-62.
174. Gibbs, M.J., J.S. Armstrong, and A.J. Gibbs, *Sister-scanning: a Monte Carlo procedure for assessing signals in recombinant sequences.* Bioinformatics, 2000. **16**(7): p. 573-82.
175. Boni, M.F., D. Posada, and M.W. Feldman, *An exact nonparametric method for inferring mosaic structure in sequence triplets.* Genetics, 2007. **176**(2): p. 1035-47.
176. Martin, D.P., et al., *RDP4: Detection and analysis of recombination patterns in virus genomes.* Virus Evol, 2015. **1**(1): p. vev003.
177. Li, H., *A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data.* Bioinformatics, 2011. **27**(21): p. 2987-93.
178. Li, H., et al., *The Sequence Alignment/Map format and SAMtools.* Bioinformatics, 2009. **25**(16): p. 2078-9.
179. Wilm, A., et al., *LoFreq: a sequence-quality aware, ultra-sensitive variant caller for uncovering cell-population heterogeneity from high-throughput sequencing datasets.* Nucleic Acids Res, 2012. **40**(22): p. 11189-201.
180. Kosakovsky Pond, S.L. and S.D. Frost, *Not so different after all: a comparison of methods for detecting amino acid sites under selection.* Mol Biol Evol, 2005. **22**(5): p. 1208-22.
181. Murrell, B., et al., *FUBAR: a fast, unconstrained bayesian approximation for inferring selection.* Mol Biol Evol, 2013. **30**(5): p. 1196-205.
182. Murrell, B., et al., *Detecting individual sites subject to episodic diversifying selection.* PLoS Genet, 2012. **8**(7): p. e1002764.
183. Weaver, S., et al., *Datamonkey 2.0: A modern web application for characterizing selective and other evolutionary processes.* Mol Biol Evol, 2018. **35**(3): p. 773-777.
184. Andrews, S. *FastQC: A quality control tool for high throughput sequence data* 2010 [cited 2021 21.12]; Available from: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.
185. Anders, S., P.T. Pyl, and W. Huber, *HTSeq--a Python framework to work with high-throughput sequencing data.* Bioinformatics, 2015. **31**(2): p. 166-9.
186. Robinson, M.D., D.J. McCarthy, and G.K. Smyth, *edgeR: a Bioconductor package for differential expression analysis of digital gene expression data.* Bioinformatics, 2010. **26**(1): p. 139-40.
187. Drummond, A.J., et al., *Bayesian phylogenetics with BEAUti and the BEAST 1.7.* Mol Biol Evol, 2012. **29**(8): p. 1969-73.
188. Rambaut, A., et al. *Tracer v1.6*. 2014; Available from: http://tree.bio.ed.ac.uk/software/tracer/.
189. Rambaut, A. *FigTree v1.4.2*. 2014; Available from: http://tree.bio.ed.ac.uk/software/figtree/.
190. Nguyen, L.T., et al., *IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies.* Mol Biol Evol, 2015. **32**(1): p. 268-74.
191. Kalyaanamoorthy, S., et al., *ModelFinder: fast model selection for accurate phylogenetic estimates.* Nat Methods, 2017. **14**(6): p. 587-589.
192. Hoang, D.T., et al., *UFBoot2: Improving the ultrafast bootstrap approximation.* Mol Biol Evol, 2018. **35**(2): p. 518-522.

193. Xi, J., et al., *Genetic characterization of the complete genome of an Aleutian mink disease virus isolated in north China.* Virus Genes, 2016. **52**(4): p. 463-73.
194. Li, Y., et al., *Genetic characterization of Aleutian mink disease viruses isolated in China.* Virus Genes, 2012. **45**(1): p. 24-30.
195. Hagberg, E.E., et al., *Evolutionary analysis of whole-genome sequences confirms inter-farm transmission of Aleutian mink disease virus.* J Gen Virol, 2017. **98**(6): p. 1360-1371.
196. Ohshima, T. and M. Mochizuki, *Evidence for recombination between feline panleukopenia virus and canine parvovirus type 2.* J Vet Med Sci, 2009. **71**(4): p. 403-8.
197. Wereszczuk, A. and A. Zalewski, *Spatial niche segregation of sympatric stone marten and pine marten--avoidance of competition or selection of optimal habitat?* PLoS One, 2015. **10**(10): p. e0139852.
198. Wereszczuk, A., R. Leblois, and A. Zalewski, *genetic diversity and structure related to expansion history and habitat isolation: stone marten populating rural-urban habitats.* BMC Ecol, 2017. **17**(1): p. 46.
199. Farid, A.H., *Response of American mink to selection for tolerance to Aleutian mink disease virus.* EC Microbiology, 2020. **16.6**: p. 110-128.
200. Bloom, M.E., et al., *Aleutian mink disease: puzzles and paradigms.* Infect Agents Dis, 1994. **3**(6): p. 279-301.

ORIGINAL PUBLICATIONS