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Diagnostics and Epidemiology of Aleutian Mink Disease Virus

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Diagnostics and epidemiology of Aleutian mink disease virus

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ACADEMIC DISSERTATION

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Contents

Abstract

Aleutian mink disease virus (AMDV) is a widespread parvovirus mainly affecting American mink (*Neovison vison*). It can cause a progressive and persistent immune complex-mediated disease (Aleutian disease, AD) in adult mink and an acute and fatal pneumonia in mink kits. The virus has a wide geographical distribution both in farmed mink and in the wild. Aleutian mink disease virus poses a major economic threat to mink farmers and it may affect the conservation and management of indigenous mustelids and other species. Infected farms are difficult to sanitize as the virus is resistant to physical and chemical treatments, it can be transmitted through several vectors and routes, and no effective medications or vaccines currently exist. Since the 1970s, diagnosis on AMDV in farmed mink has been based on the identification of specific antibodies with a counter-current immunoelectrophoresis (CIEP) test. In 2005, the Finnish Fur Breeders' Association implemented an eradication program that required the development of a new AMDV-detection protocol to screen ca. 600 000 samples per year. Although AMDV can infect and may cause disease in other mustelids and carnivores, little is known about the epidemiology and evolutionary relationships of AMDV strains in the wild in Finland and elsewhere. Thus, this study aimed to develop a modern automated test for the large-scale serodiagnosis of AMDV in mink and to elucidate the epidemiology and phylogeny of this virus in farmed mink and free-ranging mustelids in Finland.

A new antigen for the serological test was developed with a recombinant DNA technique. The *major capsid protein* (*VP2*) gene of a Finnish AMDV strain obtained from a farmed mink was amplified, cloned into a baculovirus transfer vector with subsequent recombination to baculovirus genome, and expressed in insect cells. The antigen formed virus-like particles and was confirmed to be antigenic with several serological methods. Subsequently, an enzyme-linked immunosorbent assay (ELISA) was designed for the antigen and automated. Because the small glass capillaries used to collect blood samples in CIEP could not be utilized in the ELISA test, a wicking technique using a filter paper 'blood comb' was developed. The performance of this test was compared to CIEP (an imperfect gold standard) by testing blood/serum samples from farmed mink. The results were analyzed with Bayesian modelling allowing for conditional dependence. The new automated ELISA test was found to be accurate with a diagnostic sensitivity of 96.2% (95% probability interval [PI], 91.5–99.0) and specificity of 98.4% (95% PI, 95.3–99.8), and was therefore determined suitable for the serodiagnosis of AMDV.

The epidemiology and phylogenetics of AMDV were inferred from organ and/or blood samples from farmed mink and free-ranging mustelids. The samples were screened with the newly-developed ELISA (described above) or CIEP test for anti-AMDV antibodies and with previously described or newly-developed PCR assays for AMDV DNA. Test results were studied with statistical, phylogenetic, and sequence analysis methods. Aleutian mink disease virus was found to be prevalent in the wild in Finland. A new host species, the European badger (*Meles meles*), with a prevalence of 27% (7/26; 95% confidence interval [CI], 13–46), was identified. In addition to the badger, infection markers were found in 54% (31/57; 95% CI, 42–67) of feral American mink and in one European polecat (*Mustela putorius*) (1/14; 95% CI, 1–29). No infection was found in

Eurasian otters (*Lutra lutra*) (24; 95% CI, 0–10), European pine martens (*Martes martes*) (183; 95% CI 0–1), least weasels (*Mustela nivalis*) (2; 95% CI, 0–67), stoat (*Mustela erminea*) (1; 95% CI, 0–85), or wolverine (*Gulo gulo*) (1; 95% CI, 0–85). Positive animals were distributed throughout western, southern, and eastern Finland (10/17 of sampled regions). American mink (odds ratio [OR], 335) and badger (OR, 74) had higher odds of infection compared to other species. Also, animals sampled during the first sampling period (2006–2009; OR, 5) had higher odds of infection compared to the second period (2010–2014). No significant association was detected between infection and age, sex, or region. Furthermore, mink farms were not associated with higher odds of AMDV infection nor appeared to serve as a major source of infection for free-ranging mustelids at the municipal or regional level. Based on these results, it appears that domestic and sylvatic transmission pathways are largely decoupled, but it seems probable that infections occasionally move between the farmed and wild populations (e.g., via infected escapees/intruders). A phylogenetic analysis, including Finnish, Estonian, and global strains, indicated that AMDV strains form at least five main clusters. It also inferred that the virus has been introduced to Finnish farms on at least three occasions. Unfortunately, it could not be discerned whether the occurrence of AMDV in Finland is natural or a consequence of the global mink trade. In addition to its main hosts (farmed and wild mink), similar strains of AMDV were found in pine martens, polecats, and badgers. Interestingly, Estonian badgers carried a divergent strain, possibly representing a new amdoparvovirus. Other than the strain found in Estonian badgers, and the tendency of strains from Finnish farmed and feral mink to diverge into separate clusters, AMDV strains did not cluster according to location, year, species, or pathogenicity. The nucleotide differences between Finnish AMDV sequences, based on partial *non-structural protein 1* gene, ranged from 0% to 14% and similar levels of variability were observed in farmed and natural populations.

As a result of these studies, an automated ELISA test for the serodiagnosis of AMDV was developed and validated with high diagnostic sensitivity and specificity. The test offers a low cost, easy sampling, rapid throughput of large sample numbers, reduced processing time, and automated data management. The new test can be utilized for the monitoring, control and eradication of the virus, calculating the seroprevalence, and confirming the infection status of farms or individual mink. In addition, new information on AMDV epidemiology and genetic variation in Finnish farmed mink and free-ranging mustelids were established with potential impact on the biosecurity of farms, outbreak investigations, and the conservation of threatened mustelid species. Moreover, the new diagnostic tools and additional sequence data generated in this study can be utilized in the future research on the epidemiology of AMDV.

List of original publications

This thesis is based on the following publications:

I Knuuttila A., Uzcátegui N., Kankkonen J., Vapalahti O., Kinnunen P. 2009. Molecular epidemiology of Aleutian mink disease virus in Finland. Veterinary Microbiology 133, 229–238.

II Knuuttila A., Aronen P., Saarinen A., Vapalahti O. 2009. Development and evaluation of an enzyme-linked immunosorbent assay based on recombinant VP2 capsids for the detection of antibodies to Aleutian mink disease virus. Clinical and Vaccine Immunology 16, 1360–1365.

III Knuuttila A., Aronen P., Eerola M., Gardner I.A., Virtala A.-M.K., Vapalahti O. 2014. Validation of an automated ELISA system for detection of antibodies to Aleutian mink disease virus using blood samples collected in filter paper strips. Virology Journal 11, doi: 10.1186/1743-422X-11-141.

IV Knuuttila A., Aaltonen K., Virtala A.-M.K., Henttonen H., Isomursu M., Leimann A., Maran T., Saarma U., Timonen P., Vapalahti O., Sironen T. 2015. Aleutian mink disease virus in free-ranging mustelids in Finland – a cross-sectional epidemiological and phylogenetic study. Journal of General Virology 96, 1423–1435.

The publications are referred to in the text by their roman numerals. The original articles are reprinted with the kind permission of their copyright holders.

Abbreviations

aa	amino acid
acc. no.	accession number
AD	Aleutian disease
ADE	antibody-dependent enhancement of infection
AMDV	Aleutian mink disease parvovirus
ASe	analytical sensitivity
ASM	American Society for Microbiology
ASp	analytical specificity
BMC	BioMed Central
BSA	bovine serum albumin
d_N	rate of non-silent (non-synonymous) nucleotide substitutions
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
d_S	rate of silent (synonymous) nucleotide substitutions
DSe	diagnostic sensitivity
DSp	diagnostic specificity
CI	confidence interval
CIEP	counter-current immunoelectrophoresis
CPV2	canine parvovirus type 2
CRFK	Crandell feline kidney cells
CV	coefficient of variation
EDTA	ethylenediaminetetraacetic acid
e.g.	exempli gratia, for example
ELISA	enzyme-linked immunosorbent assay
Evira	Finnish Food Safety Authority, Elintarviketurvallisuusvirasto
FPV	feline panleukopeniavirus
i.e.	id est, that is
IgG	immunoglobulin G
kDa	kilodalton
LB	Luria broth
Luke	Natural Resources Institute Finland, Luonnonvarakeskus
MEV	mink enteritis virus
ML	maximum likelihood
MP	maximum parsimony
MU	map units
NPV	negative predictive value
NS1	non-structural protein 1, major non-structural protein
NS2	non-structural protein 2
NS3	non-structural protein 3
nt	nucleotide
OD	optical density
OIE	The World Organisation for Animal Health

o/n	overnight
OR	odds ratio
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI	probability interval
p.i.	post-infection
PPV	positive predictive value
qPCR	real-time quantitative PCR
ROC	receiver operating characteristics
RT	room temperature
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Se	sensitivity
Sf9	Spodoptera frugiperda 9
SGM	Society for General Microbiology
Sp	specificity
S/P	sample to positive control
STARD	Standards for the Reporting of Diagnostic Accuracy Studies
STKL	Finnish Fur Breeders' Association, Suomen Turkiseläinten Kasvattajain Liitto
Tris	tris(hydroxymethyl)aminomethane
VLP	virus-like particle
VP1	structural protein 1
VP2	structural protein 2, major structural protein

1. Introduction

Aleutian mink disease virus (AMDV, family *Parvoviridae*) is a small and structurally simple virus with an icosahedral and non-enveloped capsid containing a short deoxyribonucleic acid (DNA) genome. Despite its apparent simplicity, the virus can cause a variety of potentially fatal disease symptoms (Aleutian disease, AD; also referred to as plasmacytosis and Sapphire disease). These range from an acute respiratory disease in kits to persistent and progressive immune complex-mediated disease in adults (reviewed by Bloom et al., 1994). Aleutian disease mainly affects American mink (*Neovison vison*), but other mustelid and carnivore species may also become infected (reviewed by Farid, 2013; reviewed by Nituch et al., 2015). The disease was first identified in farmed Aleutian genotype mink (with silver-grayish fur) in North America in the 1940s (Hartsough and Gorham, 1956), but has subsequently been found across the world in several mink-producing countries and in the wild (reviewed by Farid, 2013; reviewed by Nituch et al., 2015). Aleutian disease is an economically and ecologically important problem affecting animal welfare and health. It poses a significant financial threat to farmers (Aasted, 1985) and, potentially, indigenous wild mustelid species (Mañas et al., 2001; Yamaguchi and Macdonald, 2001; Fournier-Chambrillon et al., 2004; Nituch et al., 2012). Once a farm is infected, the disease is difficult to eradicate as the virus is transmitted through vertical, horizontal, direct and indirect routes (reviewed by Gorham et al., 1976), is resistant to many disinfectants and environmental conditions, and no effective treatment or vaccine currently exists (reviewed by Hussain et al., 2014). Currently, the prevention and control of AD in farms relies on early detection and culling of infected animals or entire herds, as well as the implementation of and adherence to effective biosecurity measures (Cho and Greenfield, 1978; Gunnarsson, 2001; Prieto et al., 2014).

Effective disease control and eradication programs rely on the development and application of new diagnostic tests[1]. When a particular test has been used for an extended period of time in a successful eradication program with decreasing prevalence, an increasing number of false-positive animals will be culled and new test systems should be developed (Thrusfield, 2007a). The detection of AMDV in mink has, since the late 1970s, mainly been based on screening for the presence of anti-AMDV antibodies using a counter-current immunoelectrophoresis (CIEP) test (Cho and Ingram, 1972). CIEP employs agarose gel electrophoresis to resolve a precipitin line formed by the antigen and the antibodies in a serum sample (Cho and Ingram, 1972). Although a simple, rapid and inexpensive method for small batches, it is not well-suited to automation and requires a large amount of labor. Published studies describing a thorough validation of this test are lacking, but estimates of diagnostic sensitivity (DSe) vary from 79% to 99% and diagnostic specificity (DSp) from 90% to 100% (Wright and Wilkie, 1982; Aasted et al., 1986; Jensen et al., 2011; Dam-Tuxen et al., 2014).

Finland is one of the major global producers of mink pelts, with approximately two million pelts with a sales value of 57 million euros in 2014 (P. Aronen [Fin Furlab], personal communication 27.5.2015). About 400 farms and 400 000 breeding mink are mainly located in western Finland (Profur, 2014). In Finland, the CIEP test has been in use

[1] *Test, assay, and method are used interchangeably in the text.*

since 1980. The mean AMDV seroprevalence of all mink tested ranged between 3% and 60% during 1980–2014, being higher during the earlier years and around 10% more recently (Kangas and Smeds, 1980s, M. Eerola [Fin Furlab], personal communication 21.5.2015). The implementation of an eradication program in 2005 by the Finnish Fur Breeders' Association (STKL) led to an increase in sample numbers from 330 000 to over 700 000 annually, with a concomitant requirement for additional labor and shortages of the CIEP antigen. Thus, an accurate, simple, economical, and high-throughput test was needed with the possibility for automation and employment of a new antigen.

In addition to farmed and feral American mink, viral DNA and/or antibodies have also been detected in several other mustelids and carnivores in Europe, North America, and Japan (Murakami et al., 2001; reviewed by Farid, 2013; reviewed by Nituch et al., 2015). Although research concerning the molecular epidemiology of various virus strains in captive and wild/feral mink has increased recently, data concerning the virus in other mustelids and carnivores remain scarce. Details concerning the clinical disease, pathogenicity, epidemiology, and phylogenetics of this virus in wild mustelids have yet to be established. The full range of host species, the prevalence of infection, and the risk factors for infection in these species are also unknown, and given that data are only available from a few countries the geographical distribution of AMDV is likely much greater than currently recognized. A few studies have indicated that infected mink farms may play a role in the transmission of the virus to the local natural population, and conversely wild or feral animals may carry the virus into farms (Oie et al., 1996; Nituch et al., 2011; Nituch et al., 2012). Further research is needed to clarify the sylvatic and domestic transmission cycles and the extent of their interaction. The origin of AMDV is unknown. Despite of its first detection and description in farmed mink of North America, it may be simple to assume that the virus originated there (Farid, 2013). However, AMDV might have existed in wild mink (and/or other mustelid) populations of North America (or elsewhere) long before its detection in farmed mink (Gorham et al., 1976). It is also possible that other mustelid species carry distinct and novel amdoparvoviruses in addition to AMDV. Notably, these viruses have already been identified in foxes (*Urocyon cinereoargenteus*, *Vulpes lagopus,* and *V. vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) (Li et al., 2011; Shao et al., 2014; Bodewes et al., 2014a).

In Finland, data on the epidemiology and phylogenetics of AMDV both in farmed mink and free-ranging[2] mustelids were practically non-existent. Potential host species, in addition to farmed and feral mink, include the seven indigenous mustelids, of which the wolverine (*Gulo gulo*) is critically endangered and the polecat (*Mustela putorius*) is considered vulnerable (Liukko et al., 2010).

The aims of these studies were to develop the serodiagnostics of AMDV and elucidate the epidemiology and phylogenetics of the virus in Finnish farmed mink and free-ranging mustelids. Specifically, the aims were to construct a new recombinant antigen with which to develop, automate, and validate a serological assay for detecting anti-AMDV antibodies in blood of farmed mink, and to develop a simple blood sampling method for this assay. Due to the potential impacts on conservation and eradication efforts, it was also important

[2] *The term free-ranging refers to both wild and feral animals in the natural environment. Feral refers to a free-ranging animal that descends from domesticated ancestors that escaped or were intentionally released and have subsequently reproduced in the wild.*

to determine whether AMDV (and/or related viruses) occurs in the Finnish natural environment and, if so, to clarify the host species, prevalence, geographical distribution, and determine any factors associated with infection. Furthermore, the origin, evolutionary relationships, and diversity of AMDV strains in farmed mink and free-ranging mustelids were studied in relation to global diversity, geographical and temporal distribution, transmission routes, pathogenicity, and the extent of genetic variation.

2. Literature review

2.1. Mink, mustelids, and mink farming

Mustelidae, comprising 57 species, is the largest family within the order Carnivora (reviewed by Larivère and Jennings, 2009). Mustelids usually have long bodies and short legs, and range in size from just 25 g for the least weasel (*M. nivalis*) to 45 kg for the sea otter (*Enhydra lutris*) (reviewed by Larivère and Jennings, 2009). They live in diverse habitats from seas and rivers to forests and grasslands. While most mustelids are terrestrial carnivores, there are also aquatic and fossorial examples (reviewed by Larivère and Jennings, 2009). Mustelids can be found on all continents except Antarctica (reviewed by Larivère and Jennings, 2009). Several species are listed as endangered by the International Union for Conservation of Nature (IUCN, 2014). Seven mustelid species are native to Finland: the European pine marten (*Martes martes*), European polecat, European badger (*Meles meles*), Eurasian otter (*Lutra lutra*), wolverine (*Gulo gulo*), least weasel, and stoat (*M. erminea*). Of these, the wolverine is critically endangered and the polecat is considered vulnerable (Liukko et al., 2010). European mink (*M. lutreola*) regrettably became extinct in Finland in the second half of the 20th century (Henttonen, 1992), and populations have also been in drastic decline elsewhere in Europe (reviewed by Maran and Henttonen, 1995). It has been speculated that the decline might in part be due to an infectious disease transmitted by the American mink, although several other possible reasons also exist (reviewed by Maran and Henttonen, 1995). The pine marten, otter, least weasel, and stoat are present throughout Finland, whereas the polecat is mainly found in the east and south-east, the badger in the south, and the wolverine in the east, west, and north (Luke, 2013; SYKE, 2014).

The semi-aquatic American mink is native to North America but has spread into several European countries, Russia, Japan, South America, and parts of Asia via the escape and/or deliberate release of farmed animals (reviewed by Reid and Helgen, 2008; reviewed by Larivère and Jennings, 2009). Farmed mink is a combination of several sub-species of the American mink: mainly Eastern (*N. vison vison*), Kenai (*N. vison melampeplus*), Alaska mink (*N. vison ingens*), and to a lesser extent the common (*N. vison mink*), Hudson Bay (*N. vison lacustris*), and Pacific mink (*N. vison energumenos*) (Shackelford, 1957). It has been farmed for its fur for decades and different traits, such as fur color (from white to black) and quality, size, and temperament, have been emphasized (Nes et al., 1988). American mink is an introduced species in Finland and the population in the wild is a result of escapees from farms and deliberate release in European parts of Russia (reviewed by Kauhala, 1996). Soon after the establishment of mink farming in Finland, the first mink were observed in the wild (Westman, 1966). In the 1950s, they were mainly present in the western and south-western coast of Finland (Westman, 1966), but today feral mink are found throughout the country (Kauhala, 1996).

Unfortunately, initial breeding trials with mink were not recorded, but the first farms were established in the 1920s in Canada, USA, and Scandinavia (Lund, 1979). In Finland, the first mink farm was established in the early 1930s (Lund, 1979). No precise records

exist concerning the trade and movement of breeding mink in Finland during the establishment of mink farming (E. Smeds [STKL], personal communication 28.9.2006; E. Puotila [Ministry of Agriculture and Forestry], personal communication 15.7.2015; P. Lappalainen [Customs], personal communication 27.7.2015), but it is probable that the first mink were imported mainly from the USA and later from Denmark and Sweden (E. Smeds [STKL], personal communication 28.9.2006). Over the years, most mink have been imported from Denmark, USA and Canada (L. Finne [STKL], personal communication 1.2.2007). Finnish farmers have exported mink mainly to Russia, Poland, and China (L. Finne [STKL], personal communication 1.2.2007). With nearly two million pelts per year (2013), Finland is one of the major global mink producing countries along with the USA, the Netherlands, Canada, Denmark, Poland, Russia, and China (Profur, 2014). Finnish pelts are produced by approximately 400 000 breeding mink housed at 400 farms (2013) (Profur, 2014). About 95% of these farms are located in four administrative regions[3] in western Finland: Ostrobothnia, North, Central, and South Ostrobothnia (A. Kettunen [STKL], personal communication, 10.6.2014). Mink are mated and the kits are born in the spring (STKL, 2015a). The winter fur develops during the fall and breeding animals for the next season are selected in the late-autumn and the remaining animals are pelted (STKL, 2015a).

2.2. Parvoviruses

The *Parvoviridae* family consists of two subfamilies: *Parvovirinae* with viruses of vertebrates; and *Densovirinae* with viruses of arthropods (Tijssen et al., 2011). The *Parvovirinae* subfamily is divided into eight genera: *Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus,* and *Tetraparvovirus* (ICTV, 2014). The diseases that parvoviruses cause in animals range from subclinical to severe gastroenteritis, myositis, myocarditis, hepatitis, pneumonia, leukopenia, and chronic immune complex disease (reviewed by Parrish, 2011). They may also cause congenital fetal anomalies and reproductive failure (reviewed by Parrish, 2011). Viruses of major veterinary importance are mainly found in the *Protoparvovirus* and *Amdoparvovirus* genera, such as feline panleukopenia virus (FPV), canine parvovirus type 2 (CPV2), mink enteritis virus (MEV), porcine parvovirus (PPV), and AMDV. Feline panleukopenia virus, CPV2, and MEV belong to the same species of *Carnivore protoparvovirus 1* (ICTV, 2014). Partly due to the advent of new molecular techniques (e.g., next-generation sequencing) several new parvovirus species, such as human parvovirus 4 (PARV4), human bufavirus, porcine hokovirus (PPV3), ovine partetravirus, canine bocavirus 2, and gray fox amdovirus, have been found in animals and humans in recent years (Li et al., 2011; reviewed by Ni et al., 2014; Yahiro et al., 2014; Bodewes et al., 2014b). However, in some cases their ability to cause disease is unclear.

Parvoviruses are small (diameter ranging from 21.5 to 25.5 nm) non-enveloped viruses with icosahedral symmetry (reviewed by Tijssen et al., 2011). The single-stranded DNA genome is linear, non-segmented, and 4–6.3 kilobases (kb) in size (reviewed by Tijssen et

[3] *Finland is divided into 320 municipalities and 19 administrative regions (see also Figure 5 for map).*

al., 2011). The palindromic sequences at both ends can form hairpin structures needed for viral replication (reviewed by Tijssen et al., 2011). Generally, parvoviruses utilize receptor-mediated endocytosis to enter the cell and various modes of trafficking within the cell (reviewed by Tijssen et al., 2011). The parvoviruses usually have two open reading frames (ORF): the left REP ORF encoding non-structural (NS) proteins and right CP ORF for structural proteins (CAP, VP, or S) (reviewed by Tijssen et al., 2011). To create different gene products from these ORFs, some parvoviruses use alternative splicing, leaky scanning and/or alternative polyadenylation (reviewed by Tijssen et al., 2011).

For replication, which takes place in the nucleus, autonomous parvoviruses require dividing cells that are going through mitotic S-phase (reviewed by Parrish, 2011; reviewed by Tijssen et al., 2011). Certain parvoviruses (genus *Dependoparvovirus*) are replication defective and need the presence of a helper virus, such as an adenovirus or herpesvirus, to replicate efficiently (reviewed by Tijssen et al., 2011; reviewed by Parrish, 2011). As fetuses and neonates have a relatively large proportion of mitotically-active cells, they are often more susceptible to diseases caused by parvoviruses (reviewed by Parrish, 2011). Thus, parvoviruses tend to have a tropism to certain rapidly-dividing cells and tissues, such as hematopoietic precursors and lymphocytes, progenitor cells of the intestinal mucosa, as well as external granular layer of the cerebellum and myocytes in the heart in early life (Parrish, 2011). Typically, parvoviruses cause permissive and acute infections (e.g. FPV, CPV2, and MEV) that last only few days, but some are able persist for long periods, even for life (e.g. AMDV, porcine parvovirus, and B19) (reviewed by Söderlund-Venermo et al., 2002; reviewed by Parrish, 2011).

2.3. Aleutian mink disease virus

Aleutian mink disease virus belongs to the species of *Carnivore amdoparvovirus 1*. Together with *Carnivore amdoparvovirus 2* (gray fox amdovirus), they are currently the sole members of the genus *Amdoparvovirus* (ICTV, 2014; Cotmore et al., 2014). However, novel amdoparvoviruses not yet listed in the International Committee on Taxonomy of Viruses (ICTV) have been recently found in arctic and red foxes and raccoon dogs (Shao et al., 2014; Bodewes et al., 2014a).

2.3.1. Morphology, genome, and proteins

Aleutian mink disease virus has a non-enveloped and icosahedral virion about 25 nm in diameter (reviewed by Cho, 1976). The single-stranded DNA genome is approximately 4.7 kb in size with terminal hairpin structures and negative polarity (Bloom et al., 1990). The genome contains three ORFs which encode for five proteins: ORF1 encodes NS1 protein with molecular weight of 70 kilodaltons (kDa); ORF2 encodes NS3 (10 kDa); and ORF3 encodes NS2 (17 kDa), structural proteins VP1 (85 kDa) and VP2 (75 kDa) (Qiu et al., 2006; Qiu et al., 2007; Huang et al., 2014). Six messenger RNAs (mRNA) are generated from one promoter (P3) by alternative splicing and polyadenylation (Qiu et al.,

2006). The non-structural proteins share 60 amino acids at the N (amino)-terminus (Huang et al., 2014). The VP1 sequence contains the entire VP2 sequence with additional 44 amino acids in its N-terminus (Christensen et al., 1993).

In parvoviruses, the NS1 protein has several functions associated with viral replication, regulation of DNA replication, transcription and packaging of viral DNA, release and spread of virus progeny, transactivation of viral and cellular genes, DNA damage response, and enzymatic activities (nickase, helicase/ATPase) (reviewed by Tewary et al., 2014; reviewed by Nuesch and Rommelaere, 2014). It also plays a role in the cell cycle arrest, modulation of host innate immunity, infectivity, cytotoxicity, and apoptosis (reviewed by Tewary et al., 2014; reviewed by Nuesch and Rommelaere, 2014). In AMDV, the NS1 protein is presumed to have similar functions, but it may also be involved in the restriction of virus replication and pathogenicity (Bloom et al., 1982; Huang et al., 2014). The smaller non-structural proteins, NS2 and NS3, are required for viral replication (Huang et al., 2014). The AMDV strains share over 87% nucleotide (nt) and 82% amino acid (aa) identities in the *NS1* gene (Li et al., 2011). However, a higher degree of variability, up to 19% at the nt and 30% at the aa level, are seen within certain regions (Olofsson et al., 1999). The middle region of the protein is more conserved than the N- and C (carboxy)-termini (Gottschalck et al., 1994).

The AMDV capsid is formed by VP1 and VP2 proteins. It contains 60 protein particles from which 10% are VP1 and 90% VP2 (McKenna et al., 1999). The *VP2* gene seems to be more conserved than *NS1* with over 92% nt and 91% aa identities (Li et al., 2011). Certain regions show a divergence of up to 11% at the nt and 15% at the aa level (Oie et al., 1996; Nituch et al., 2012). Variable nt positions are clustered within an area known as the hypervariable region at nt 690–730, MU 64–65 (Gottschalck et al., 1991; Gottschalck et al., 1994; Oie et al., 1996). Generally in parvoviruses, the capsid surface has a major role in determining the pathogenicity, along with the antigenicity, host range, and cellular tropism (McKenna et al., 1999; reviewed by Kontou et al., 2005). It appears that a particular region of the AMDV VP2 sequence (residues 428–446 in the icosahedral twofold depression) is involved in the pathogenesis of AD, i.e., immune complex formation and antibody-dependent enhancement of infection (ADE) (Bloom et al., 2001). In addition to this site, other specific regions in the capsid proteins (e.g., valine residue at codon 352, aspartic acid at 534, residues in the two- and threefold axes, map units [MU] 54–65, MU 64–65, and MU 65–88) may contribute to the pathogenicity, replication, and/or determination of the host range (Bloom et al., 1988; Gottschalck et al., 1991; Bloom et al., 1993; Bloom et al., 1998; Fox et al., 1999; McKenna et al., 1999; Bloom et al., 2001; McCrackin Stevenson et al., 2001).

2.3.2. Replication and persistence

Aleutian mink disease virus is an autonomous parvovirus, i.e., replication can take place without the need for a helper virus. Replication is restricted in adult mink, which creates a persistent and non-cytopathic infection (Alexandersen et al., 1988; Alexandersen et al., 1989). Cytopathic and permissive replication is known to occur only in seronegative mink kits (in type II pneumocytes) and in Crandell feline kidney (CRFK) cells (Bloom et al.,

1980; Alexandersen et al., 1987; Alexandersen and Bloom, 1987; Alexandersen et al., 1989). The primary sites for replication in adults are B-lymphocytes, macrophages, and dendritic cells in lymphoid organs (primarily lymph nodes and spleen) (Alexandersen et al., 1988; Wohlsein et al., 1990; Mori et al., 1991; Aasted and Leslie, 1991; Kanno et al., 1992). Some replication may also occur in the blood leukocytes and bone marrow of adults and in the liver and kidney of kits (Alexandersen et al., 1987; Alexandersen et al., 1988). The entry of AMDV into target cells is likely to occur via ADE, where virus-antibody complexes bind to cellular Fc-receptors that facilitate viral entry into the cell (Kanno et al., 1993; Bloom et al., 2001). Mechanisms for viral persistence and restricted replication appear to be associated with non-neutralizing antibodies, ADE, functions of the non-structural proteins, restriction of capsid protein production by caspase cleavage, and internal polyadenylation of pre-mRNA (Kanno et al., 1993; Alexandersen et al., 1994a; reviewed by Best and Bloom, 2005; Cheng et al., 2010; Huang et al., 2012). The permissive replication in CRFK cells seems to be facilitated by apoptosis and caspase activity (particularly caspase-3) (Best et al., 2002).

In kits and adults, the level of viral replication typically peaks 9 to 14 days post-infection (p.i.) (Porter et al., 1969; Alexandersen and Bloom, 1987; Alexandersen et al., 1988), after which the number of cells producing the virus decreases considerably (Alexandersen et al., 1988). The virus can first be detected from 4 to 14 days p.i., primarily in the spleen and lymph nodes of adults and in the lungs of kits, but to some extent (mainly in the form of sequestered virion DNA) also in the intestine, kidney, bone marrow, liver, blood, plasma/serum, and brain (Gorham et al., 1964; Eklund et al., 1968; Hadlow et al., 1985; Alexandersen et al., 1987; Alexandersen et al., 1988; Oie et al., 1996; Jahns et al., 2010; Jensen et al., 2014; Farid et al., 2015). Even in asymptomatic mink, the virus can persist in tissues for several months, possibly for life (Eklund et al., 1968; Hadlow et al., 1985; Oie et al., 1996), although it seems that some non-Aleutian mink are able to clear the virus (Hadlow et al., 1984). Viremia develops five days to four weeks p.i. and appears to persist in Aleutian mink infected with highly pathogenic strains, whereas mink (especially non-Aleutian) infected with low pathogenicity strains may develop only transient or intermittent viremia (Eklund et al., 1968; Hadlow et al., 1985; Oie et al., 1996; Jackson et al., 1996a; Jensen et al., 2014). Following an antibody response, the viremia seems to decrease (Jackson et al., 1996a). However, the onset of infection and viremia, and the level of virus replication vary greatly among individuals (Hadlow et al., 1985).

2.3.3. Propagation and cultivation

Aleutian mink disease virus is propagated in cell culture or in mink. However, only a few strains (e.g., AMDV-G, -P, and -GL) are adapted to grow in cell culture, more specifically in feline renal epithelial (CRFK) cells at 31.8 °C (Porter et al., 1977b; Bloom et al., 1980; Alexandersen, 1990). Most of the cultivable strains originate from the highly pathogenic Utah 1 strain (Bloom et al., 1980). Of these the non-pathogenic AMDV-G, isolated in the late 1970s, has become the most widely used for research and diagnostic purposes as it grows to higher titers than most other strains (Bloom et al., 1980). After several passages in cell culture, AMDV-G has lost its pathogenicity to mink (Bloom et al., 1980; Oie et al.,

1996). As the cultivation of field strains in cell culture is often unsuccessful (reviewed by Porter et al., 1977b), most AMDV strains are propagated in live mink. The mink are usually infected with intraperitoneal injections and euthanized ten days p.i. (reviewed by Cho, 1976).

2.3.4. Different strains and their pathogenicity

Aleutian mink disease virus strains can be categorized roughly into four classes based on their pathogenicity[4] to mink: non, low, moderate, and high (Table 1). All strains infect both Aleutian and non-Aleutian mink, some strains only cause disease in Aleutian mink whereas others induce severe symptoms and lesions in mink of all genotypes (Hadlow et al., 1983). It seems that strains do not differ in the severity (i.e., virulence) of the disease they cause; rather, the genotype of the mink plays a more important role. The final level of hypergammaglobulinemia and terminal pathology seem to be similar in different strains (Hadlow et al., 1983; Alexandersen et al., 1994b). However, highly pathogenic strains may induce higher levels of hypergammaglobulinemia and severe lesions sooner (Hyllseth et al., 1992; Alexandersen et al., 1994b), thus meaning a faster progression. Field strains appear to be mostly of low to moderate pathogenicity (Gorham et al., 1976; Hadlow et al., 1983; Porter, 1986), but occasionally severe, rapidly spreading outbreaks occur, such as in the case of AMDV-K and -TR (Henson et al., 1976; Alexandersen, 1990; Oie et al., 1996). The determinants of AMDV pathogenicity have been extensively studied (see Chapter 2.3.1) and although some have been identified, mainly in specific regions of the capsid, they seem to be complex and in need of further research.

No official strain-demarcation criterion currently exists for AMDV and all isolates seem to be similar antigenically (reviewed by Tijssen et al., 2011). The classification in Table 1 is mainly based on clinicopathologic observations and later on sequence data. Serologically, strains are closely related (Aasted et al., 1984a). Gottschalck et al. (1991) proposed a typing scheme based on the amount of nucleotide differences between strains, where strains with minor differences were of the same type and with major differences of different type. AMDV-G, -SL3, and -TR were type 1, -Utah 1 type 2, -K type 3, and -United type 4 (Gottschalck et al., 1991; Gottschalck et al., 1994; Oie et al., 1996; Schuierer et al., 1997). However, this typing scheme was based on only a few strains, does not correlate with pathogenicity (Oie et al., 1996; Schuierer et al., 1997), and no objective limits were proposed for the amount of nucleotide differences between different types. More recently, Christensen et al. (2011) used a molecular typing scheme for categorizing Danish strains based on differences in the *NS1* gene. Whether this can also be used on a global scale remains to be determined.

[4] *Pathogenicity, ability of the organism to cause disease; virulence, severity of the disease caused by the organism.*

Table 1. *Background information on some of the AMDV strains used for research and/or diagnostic purposes*

Strain	Pathogenicity	Clinical disease	Source	Year of isolation	Region, country	Reference
AMDV-G	None	No	Cell culture adapted clone of Utah 1	Late 1970s	USA	(Bloom et al., 1980; Oie et al., 1996)
AMDV-Pullman	Low	Mainly in Aleutian mink	Spleen	1961	Washington, USA	(Gorham et al., 1964; Bloom et al., 1975; Hadlow et al., 1983)
AMDV-F	None in mink; low in ferret	In ferrets	Spleen of a ferret	Early 1980s	USA	(Porter et al., 1982)
AMDV-P	Moderate	In Aleutian and some non-Aleutian mink	Cell culture clone of Utah 1	1970s	USA	(Porter et al., 1977b; Bloom et al., 1980)
AMDV-Montana	Moderate	In Aleutian and some non-Aleutian mink	Spleen	1973	Montana, USA	(Hadlow et al., 1983)
AMDV-SL3	Moderate	In Aleutian mink	Bone marrow	Early 1980s	Germany	(van Dawen et al., 1983; Haas et al., 1990; Schuierer et al., 1997)
AMDV-GL	Variable	None in adult mink; pneumonia in kits	Cell culture isolate derived from AMDV-G	1980s	Denmark	(Alexandersen, 1990)
AMDV-Utah 1	High	In all mink genotypes	Spleens of 30 mink	1963	Utah, USA	(Porter et al., 1969; Hadlow et al., 1983)
AMDV-K	High	In all mink genotypes	Lungs, livers, and spleens of 40 mink kits	1982	Denmark	(Alexandersen, 1986; Alexandersen, 1990)
AMDV-Ontario	High	In all mink genotypes	Spleen, liver, and kidney	1961	Ontario, Canada	(Hadlow et al., 1983)

Strain	Pathogenicity	Clinical disease	Source	Year of isolation	Region, country	Reference
AMDV-TR	High	In all mink genotypes	Mesenteric lymph nodes of infected mink	1990s	Utah, USA	(Oie et al., 1996)
AMDV-United	High	In all mink genotypes	Was thought to contain Utah 1 strain	ND	Madison, USA	(Gottschalck et al., 1994)

Abbreviations: AMDV, Aleutian mink disease virus; ND, no data. Modified from Knuuttila (2007).

2.3.5. Transmission

Aleutian mink disease virus takes advantage of multiple modes of transmission. Mink may become infected vertically through placenta, or horizontally via indirect and direct transmission pathways (Padgett et al., 1967; reviewed by Gorham et al., 1976; Porter et al., 1977a; Broll and Alexandersen, 1996). Aleutian mink disease virus can be found in the feces, urine, blood, serum, and saliva of infected animals (Gorham et al., 1964; Jensen et al., 2014; Farid et al., 2015). Horizontal transmission occurs via peroral and saliva-aerosol-respiratory routes either in direct contact with an infected mink (or other animals) or in indirect contact with contaminated feed, water, air, pens, equipment, and vehicles (Gorham et al., 1964; reviewed by Gorham et al., 1976; Jackson et al., 1996a; Jackson et al., 1996b). Infection may also be transmitted by iatrogenic route (e.g., toenail clippers), biting, and possibly insects (reviewed by Gorham et al., 1976; Cho and Greenfield, 1978; Jackson et al., 1996a). Airborne transmission has also been suggested as a mode of transmission between farms (Jackson et al., 1996b), but this has not yet been verified (reviewed by Jensen et al., 2014) and its role is probably unimportant (Christensen et al., 2011; Espregueira Themudo et al., 2012).

The rate of transmission within and between farms may vary considerably depending on the pathogenicity of the virus strain (Gorham et al., 1964; Gorham et al., 1976; Cho and Greenfield, 1978; Oie et al., 1996) and the genotype of mink being raised (Cho and Greenfield, 1978). Thus, even within one farm the seroprevalence might be as high as 80% in one shed and less than 4% in another (Cho and Greenfield, 1978). AMDV strains of low pathogenicity seem less easily transmitted, as mink caged next to an infected individual or even experimentally-inoculated animals may remain uninfected (Gorham et al., 1964; Eklund et al., 1968; Bloom et al., 1975; Gorham et al., 1976; Cho and Greenfield, 1978; Hadlow et al., 1983; Oie et al., 1996; Jensen et al., 2014), or it may take three to four weeks for the infection to transmit from one cage to another (Jensen et al., 2014). However, highly pathogenic strains may cause severe, rapidly spreading outbreaks where the herd of an AMDV-free farm can become 90% infected in less than six months (Alexandersen, 1990; Oie et al., 1996). It should be noted that farms and even individual mink may be infected with multiple strains (Hadlow et al., 1984; Gottschalck et al., 1991; Olofsson et al., 1999; Jahns et al., 2010).

Research on the sylvatic transmission cycle and the transmission between farms and the wild has been scarce, but increasing in recent years. Some infer that wild/feral animals have separate cycles (Leimann et al., 2015), whereas others show that cross-infections between wild and captive populations do occur (Oie et al., 1996; Nituch et al., 2011; Nituch et al., 2012; Nituch et al., 2015).

2.4. Aleutian disease

2.4.1. History

The first case of AD was identified in 1946 in North America (Hartsough and Gorham, 1956). It was initially described in mink with Aleutian (silver-grayish) coat color, named after Aleutian fox of a similar color. Viral etiology was proposed in the early 1960s (Karstad and Pridham, 1962; Trautwein and Helmboldt, 1962) and in 1974, a parvovirus was suggested as the causative agent of AD (Cho and Ingram, 1974). It is possible that the emergence of a new and more susceptible host (i.e., the Aleutian genotype mink with lysosomal abnormality) brought about the clinical disease (Gorham et al., 1976). However, the virus might have been present in mink farms and in the wild before the disease emerged (Gorham et al., 1976). In the late 1950s and early 1960s, AD was also found in Sweden and Denmark (reviewed by Aasted, 1985). Today, it is present in many mink-producing countries (reviewed by Aasted, 1985) and the virus has also been detected in natural populations of mustelids (reviewed by Farid, 2013; reviewed by Nituch et al., 2015). Initially, AD was believed to only affect Aleutian mink, but it was soon realized that all genotypes (e.g., standard dark, black, and pastel) were susceptible (reviewed by Aasted, 1985). Of the farmed genotypes, Aleutian mink are more prone to develop a more serious form of AD (Eklund et al., 1968; Bloom et al., 1975; Larsen and Porter, 1975; Hadlow et al., 1983; Oie et al., 1996). Costs to farmers and the industry include the loss of breeding animals and kits, infertility, reduced litter sizes, abortions, low-quality fur, restrictions to international trade, and costs related to control measures (Eklund et al., 1968; Aasted, 1985; Alexandersen, 1986). In Denmark, losses to the mink industry were estimated to be approximately 10 million dollars per year (Aasted, 1985). In Finland, the annual costs are approximately from two to three million euros (P. Aronen [Fin Furlab], personal communication 27.5.2015).

2.4.2. Pathogenesis

Aleutian disease manifests along a severity continuum depending on viral (strain and dose) and host (immune status, age, and genotype) factors. The disease outcomes can be categorized as follows: 1) progressive, persistent, and fatal (‘classical AD’); 2) non-progressive and persistent; 3) transient; and 4) acute fatal pneumonia in mink kits (Figure 1) (reviewed by Bloom et al., 1994). Progressive disease is characterized by high antibody titers, hypergammaglobulinemia, and lesions induced by immune complexes; non-progressive disease by lower antibody titers, lower levels of or no hypergammaglobulinemia, and no lesions; and transient by lower and decreasing antibody titers and no viremia with potential virus clearance (Larsen and Porter, 1975; Henson et al., 1976; Hadlow et al., 1984; reviewed by Porter, 1986; reviewed by Jackson et al., 1996a). As much as 25% of non-Aleutian mink may experience transient infections (Larsen and Porter, 1975).

In adult mink, AMDV causes a disease that is different from what is typically seen in parvoviruses. The clinical signs and lesions are actually a result of the host immune response to the virus, rather than being caused by direct actions of the virus (reviewed by Porter, 1986). Aleutian disease develops when the circulating antigen-antibody complexes deposit in tissues and cause type III hypersensitivity reaction (reviewed by Parrish, 2011). The monocytic cells, consisting mainly of plasma cells, and immune complexes accumulate in the kidneys, liver, spleen, bone marrow, and arteries causing tissue injury (reviewed by Porter et al., 1980).

Mink homozygous for the *Aleutian* gene (*aa*), and color crosses such as Sapphire (*aapp*) and Violet (*aammpp*), are more susceptible to AD (Eklund et al., 1968; Bloom et al., 1975; Hadlow et al., 1983; Anistoroaei et al., 2013). The diluted pigmentation of the fur is a result of a lysosomal disorder (Chédiak-Higashi syndrome) caused by a mutation in the *lysosomal trafficking regulator* (*LYST*) gene (Anistoroaei et al., 2013). Thus, a more rapid progression of the disease and higher mortality rates compared to non-Aleutian mink (Eklund et al., 1968; Bloom et al., 1975; Larsen and Porter, 1975; Hadlow et al., 1983) may be due to the syndrome (Anistoroaei et al., 2013).

In seronegative mink kits less than three weeks old, the disease is acute and caused by viral injury to type II pneumocytes resulting in fatal interstitial pneumonia (Alexandersen, 1986; Alexandersen et al., 1994b). Both strains of high and low pathogenicity can induce pneumonia, but with varying morbidity and mortality (Alexandersen, 1986; Alexandersen et al., 1994b). Kits that survive the infection develop classical AD (Alexandersen, 1986; Alexandersen et al., 1994b). Although antibodies are unable to neutralize AMDV, they restrict viral replication in type II pneumocytes (Alexandersen, 1986; Alexandersen et al., 1989). Thus, kits with antibodies received from a seropositive dam or kits infected transplacentally do not develop pneumonia (Porter et al., 1977a; Alexandersen, 1986; Alexandersen et al., 1989). Instead, AMDV infection in these kits results in a prolonged and non-progressive AD with mild lesions (Porter et al., 1977a; Alexandersen, 1986; Alexandersen et al., 1989).

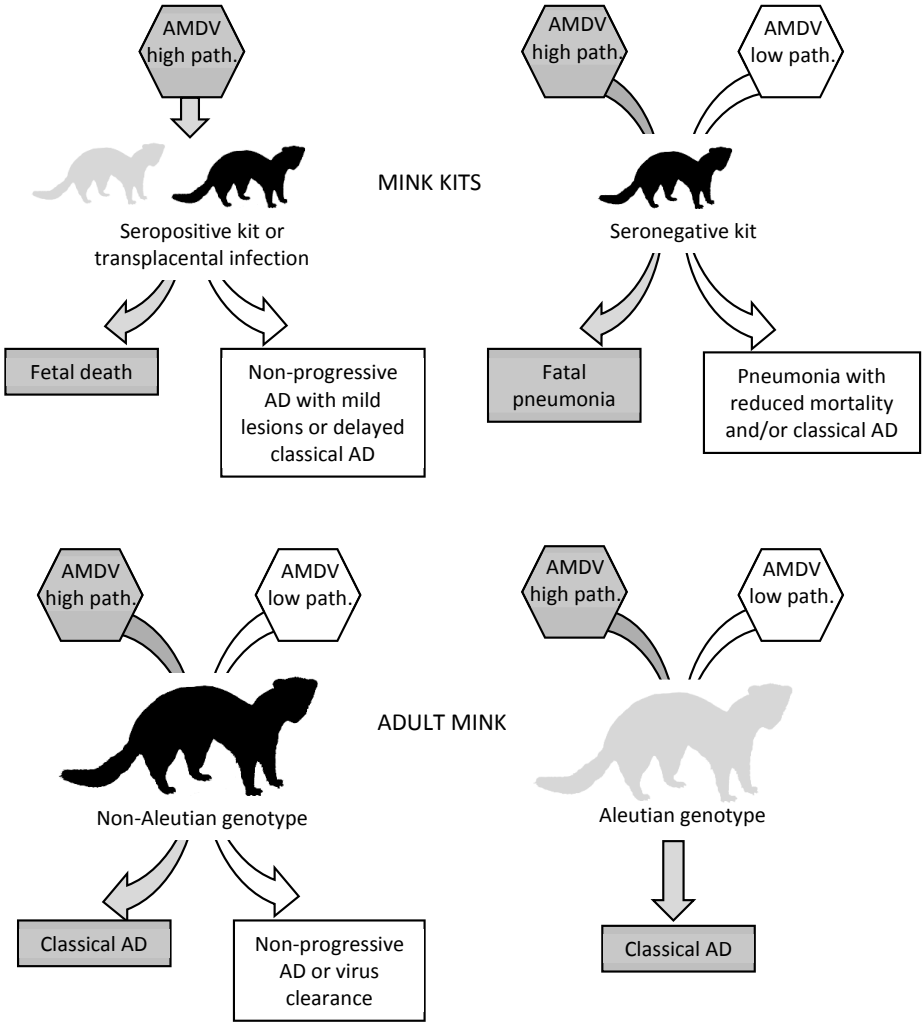

Figure 1. *Disease outcomes of Aleutian disease related to virus strain, mink genotype (Aleutian, gray; non-Aleutian, black), and age.* Abbreviations: AMDV; Aleutian mink disease virus, AD; Aleutian disease. Modified from (Knuuttila, 2007). Compiled from the following sources: (Larsen and Porter, 1975; Henson et al., 1976; Porter et al., 1977a; Hadlow et al., 1983; Hadlow et al., 1984; Alexandersen, 1986; Alexandersen and Bloom, 1987; Alexandersen et al., 1989; Alexandersen et al., 1994a; Alexandersen et al., 1994b; Oie et al., 1996).

2.4.3. Antibody response

During AMDV infection, high levels of antibodies are produced and target structural and non-structural proteins, predominantly conformational epitopes (Bloom et al., 1982; Aasted and Bloom, 1984; Costello et al., 1999). Although abundant, these antibodies are unable to neutralize the virus (reviewed by Porter, 1986) and infection with one strain provides little protection to future infections by other strains (Hadlow et al., 1984). Both mink genotypes produce high-affinity antibodies, but in non-Aleutian mink the quality of the antibodies decreases and they become more heterogeneous as the disease progresses (Aasted and Bloom, 1984). Although highly pathogenic strains can induce high levels of hypergammaglobulinemia and production of anti-AMDV antibodies more rapidly than do strains of low pathogenicity (Hyllseth et al., 1992), the affinity of the antibodies seems to be lower (Aasted and Bloom, 1984). A progressive disease is related to higher antibody titers, higher hypergammaglobulinemia, and more severe lesions; whereas a non-progressive disease is characterized by lower antibody titers with no hypergammaglobulinemia or lesions (Larsen and Porter, 1975; Henson et al., 1976; reviewed by Porter, 1986; reviewed by Jackson et al., 1996a). In kits, low or no production of antibodies is linked to permissive and high levels of replication resulting in an acute and severe disease, while a chronic disease develops where replication is restricted to a low level and antibody production is high (Alexandersen et al., 1989).

Increased total serum protein, gammaglobulin, and IgG are evident from 15 to 18 days p.i. (Porter et al., 1984b). The first anti-AMDV antibody to appear is IgM, which starts to increase six days p.i. with low levels present in most mink for at least 85 days p.i. (Porter et al., 1984b). Also, low levels of AMDV-specific IgA can be found throughout the infection period (Porter et al., 1984b). Most of the gammaglobulin and anti-AMDV antibody is IgG, which is present by day 15 p.i. and rises throughout the disease process (Porter et al., 1984b; Aasted et al., 1984b). The increase in gammaglobulin is mainly a result of increased amounts of AMDV-specific antibodies, but anti-DNA antibodies are also produced (Henson et al., 1976; Hahn and Hahn, 1983; Porter et al., 1984a; Aasted et al., 1984b). Aleutian mink tend to have higher gammaglobulin and IgG levels (Porter et al., 1984b) and the antibody response in this genotype invariably leads to fatal disease contrary to non-Aleutian mink (Hadlow et al., 1983). Thus, in non-Aleutian mink, especially when infected with strains of low pathogenicity, the hypergammaglobulinemia may be transient and the level of AMDV-specific antibodies is lower and decreasing (Eklund et al., 1968; Larsen and Porter, 1975; Bloom et al., 1975; Henson et al., 1976; Hadlow et al., 1983; Aasted et al., 1984b). Regardless of this, antibodies persist for several years and possibly for life (Hadlow et al., 1983).

2.4.4. Clinical signs and lesions

Severity of clinical signs and the rate at which the disease develops varies greatly among individual mink (Eklund et al., 1968). Generally, the disease is more severe and the progression is faster in mink kits with pneumonia and in Aleutian mink (Eklund et al., 1968; Henson et al., 1976; Hadlow et al., 1983; Alexandersen et al., 1994b), which usually

die after a few days (Alexandersen and Bloom, 1987) and from two to six months p.i., respectively (Eklund et al., 1968). Non-Aleutian mink, although persistently infected, rarely die before five months and may survive for several years up to a full life span (i.e., eight years) (reviewed by Porter et al., 1980; Hadlow et al., 1983). The first lesions in adults (plasmacytosis and lymphadenopathy) appear two weeks p.i. (Bloom et al., 1994) and in mink kits five days p.i. (Alexandersen and Bloom, 1987). Death in adults is often caused by renal failure or hemorrhageing (Eklund et al., 1968; Henson et al., 1976; reviewed by Porter et al., 1980). In addition to host and viral factors, environmental and physiological determinants may also have a substantial effect on the mortality rate associated with AD in mink farms (Eklund et al., 1968; Gorham et al., 1976). Table 2 presents the clinical signs and gross and histopathologic lesions of AD in mink kits and adult mink.

Table 2. *Clinical signs, gross, and histopathologic lesions of Aleutian disease in adult mink and mink kits*

Disease type	Incubation period[a]	Clinical signs	Duration[b]	Laboratory findings	Gross lesions	Histopathologic lesions	Morbidity[c]	Mortality[c]
AD in adults	1–3 mo	Lethargy, anorexia, cachexia, polydipsia, low quality fur, reproductive failure, neurological symptoms, melena, ulcers and bleeding in mucosal surfaces, dehydration.	1.5 mo–3.5 y	Hypergamma-globulinemia, high anti-AMDV antibody levels, infectious immune complexes, viremia, anemia, uremia, thrombocytopenia, clotting abnormalities.	Lymphadenopathy, splenomegaly, hepatomegaly, enlarged pale kidneys, CNS and mucosal hemorrhages, subcutaneous edema, ascites.	Plasma cell-dominated mononuclear cell infiltrates in the kidneys, liver, spleen, lymph nodes, bone marrow, arteries, and brain and meninges; immune complex vasculitis, arteritis with fibrinoid necrosis, uveitis, and glomerulonephritis; proliferation and dilatation of bile ducts.	0–100%	0–100%
Pneumonia in seronegative kits	9–20 d	Respiratory distress, abdominal respiration, costal retractions, grunting, lethargy.	9–21 d		Interstitial pneumonia, reddening, meaty consistency, edema.	Hyperplasia and -trophy of type II pneumocytes, interstitial edema, hyaline membrane formation, thickening of interalveolar septa, intra-alveolar hemorrhages and accumulation of macrophages and cellular debris, INIBs in type II pneumocytes.	Highly pathogenic strains 90–100%; low pathogenic 50–70%.	Highly pathogenic strains 90–100%[d]; low pathogenic 30–50%.

[a] From inoculation/infection to the onset of clinical signs.
[b] From inoculation/infection to death.
[c] Depends on the pathogenicity of the infecting/inoculated virus strain and its dose and age, immune status and genotype of the host.
[d] The presence of antibodies reduces the mortality to 25–50%.
Abbreviations: AD; Aleutian disease, INIB; intranuclear inclusion body, CNS; central nervous system.
Compiled from the following sources: (Padgett et al., 1967; Eklund et al., 1968; Bloom et al., 1975; reviewed by Henson et al., 1976; reviewed by Porter et al., 1980; Hadlow, 1982; Hadlow et al., 1983; Alexandersen, 1986; Alexandersen and Bloom, 1987; Alexandersen et al., 1989; Alexandersen et al., 1994b; Oie et al., 1996; Jahns et al., 2010).

2.4.5. Control, prevention, and treatment

Currently, no legistlation exists for the control of AMDV (Decree of the Ministry of Agriculture and Forestry on Controlling Animal Diseases and Their Classification[5], 843/2013) in Finland and although it is not notifiable to the World Organisation for Animal Health (OIE, 2015), it is notifiable (Decree of the Ministry of Agriculture and Forestry on Reporting of Animal Diseases and Supplying of Microbial Strains[5], 1010/2013) to the Finnish authorities. Given that prevention and control are not mandated by government, they have been organized by the industry and its representative, the Finnish Fur Breeders' Association (STKL). To improve animal health and welfare, and to reduce the financial losses to farmers due to the reproductive failure, smaller litters, loss of breeding animals, and low quality fur, STKL executed an AMDV eradication program in 2005. Finnish Fur Breeders' Association provides farmers with financial and advisory support to implement the control measures. Farms are categorized into groups A–E based on AMDV seroprevalence (STKL, 2015b). All listed farms test their breeding animals once or twice annually from January to February and/or from May to June (STKL, 2015b). Group A-farms have zero test prevalence (Aronen, 2006). Group B-farms should not have more than 1 positive/1000, group C 2/1000, and group D 50/1000 breeding females (Aronen, 2006). Group E-farms have more than 50 positive/1000 breeding females (Aronen, 2006).

The main strategies employed in the eradication program are the identification and culling of infected animals (test-and-remove) or culling the entire herd (stamping out) with thorough cleaning and disinfection of the premises, repopulation with AMDV-free animals, and strict biosecurity measures (Cho and Greenfield, 1978; Gunnarsson, 2001; Prieto et al., 2014). Of major importance is the prevention of reinfection, as infected farms are difficult to sanitize due to the stability of the virus in the environment, its multiple modes of transmission, persistent infections, and the lack of effective therapy. New outbreaks may occur due to inadequate disinfection of the premises or through contact with nearby infected farms (Themudo et al., 2011; Christensen et al., 2011; Espregueira Themudo et al., 2012; Farid et al., 2012). Thus, cooperation between farms is imperative for effective results. Other important points for AMDV control include replacement animals, visitors, shared workers, vehicles, feed, manure, equipment, and wild/feral animals (Oie et al., 1996; Gunnarsson, 2001; Christensen et al., 2011; Farid et al., 2012; Espregueira Themudo et al., 2012; Prieto et al., 2014). It seems that test-and-remove strategy, although effective in reducing the prevalence, has so far failed to eradicate the virus from infected farms (Farid et al., 2012). Many mink-producing countries (e.g., Denmark, Finland, Canada, and Iceland) have implemented programs to eradicate AMDV and have been able to reduce prevalence and sanitize individual farms and areas (Gunnarsson, 2001; Farid et al., 2012; Espregueira Themudo et al., 2012). However, the strict stamping-out policy employed in Iceland offers the only example of a strategy that

[5] *Unofficial translations from Finnish: Maa- ja metsätalousministeriön asetus vastustettavista eläintaudeista ja niiden luokittelusta MMMa 843/2013 and Maa- ja metsätalousministeriön asetus eläintautien ilmoittamisesta ja mikrobikantojen toimittamisesta MMMa 1010/2013.*

has successfully eliminated the virus from farms (Gunnarsson, 2001). The Danish program has also been succesful and positive-testing farms are now confined to northern Denmark (Espregueira Themudo et al., 2012). In Finland, both strategies are applied and the eradication has been successful in individual farms and areas, which has led to an increase in the proportion of AMDV-free farms; from 32% in 2005 to 48% in 2014 (P. Aronen [Fin Furlab], personal communication 27.5.2015; M. Eerola [Fin Furlab], personal communication 21.5.2015). However, this improvement is against a backdrop of increasing mean seroprevalence (3% to 15% since 2006) (M. Eerola [Fin Furlab], personal communication 21.5.2015) probably brought about by the implementation of more extensive testing of heavily infected farms.

Inactivation of AMDV is challenging. As a typical parvovirus, it is able to survive a variety of physical and chemical treatments, e.g., ether, fluorocarbon, pH 3, deoxycholic acid (a bile acid), protease and nuclease digestion, 1% chloramine, 2% 2-phenylphenol, and various heating protocols ranging from 56 °C for 30 min to 99.5 °C for 3 min (reviewed by Cho, 1976; Eterpi et al., 2009; reviewed by Hussain et al., 2014). On the other hand, AMDV is sensitive to 0.05 M NaOH, 0.5 M HCl, 0.5% iodine, 0.3% formalin treatment for 8 h, ultraviolet light, and heating to 80 °C for 24 h or 65 °C for three days (reviewed by Cho, 1976; Hussain et al., 2014). In the field conditions, AMDV can be inactivated by composting at 65 °C for four days (Hussain et al., 2014). Farm equipment and surfaces are typically disinfected with agents containing glutaraldehyde or oxidative substances (e.g., Virkon S or Parvocide), gas flame, and/or formalin gassing (Kankkonen, 2006).

Effective medical treatment and an AMDV vaccine are currently lacking. Trials to develop an inactivated virus or a VP1/VP2 protein vaccine have been unsuccessful, as the antibodies induced by the vaccine tend to generate a more severe disease and actually increase mortality once the animal is challenged with the live virus (Porter et al., 1972; Aasted et al., 1998). For the same reason, treatment with passive antibodies has failed (Porter et al., 1972). Mink immunized with NS1 protein and NS1 DNA vaccines (or a combination of both) exhibit less severe symptoms when challenged but only partial protection is achieved (Aasted et al., 1998; Castelruiz et al., 2005). Although AD can be treated with immunosuppressive or immunomodulatory drugs such as cyclophosphamide or corticosteroids, these have not been used in mink farming due to their high cost (Cheema et al., 1972). Thus, medical treatment and supportive care is usually applied only to important breeding animals and pets such as ferrets. Despite the treatment, the prognosis of AD is poor (Hillyer and Brown, 2000).

The notion that some mink are resistant to infection is uncertain (Hadlow et al., 1983). Breeding programs that have crossed non-affected genotypes to improve AMDV resistance have been unsuccessful (Larsen and Porter, 1975; Henson et al., 1976). More recent results suggest that there may be some hereditary differences in susceptibility to AMDV infection, although heritability is low (Häkli, 2013).

2.5. Diagnostics

2.5.1. Diagnostic tests and their accuracy

Diagnostic tests of viral infections can be based on the identification of the virus, viral antigens, viral nucleic acids, or host antibodies (reviewed by MacLachlan and Dubovi, 2011). Different serological methods can be used to detect virus-specific antibodies, such as enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence, serum neutralization, hemagglutination inhibition, complement fixation, Western blotting, and immunodiffusion (reviewed by MacLachlan and Dubovi, 2011). Given the large number of animals that must be screened, simple, cost efficient, and rapid tests with high DSe and DSp are required (reviewed by MacLachlan and Dubovi, 2011). ELISA tests that identify virus-specific antibodies and polymerase chain reaction (PCR) tests that detect viral nucleic acids are standard methods for diagnosing infections today (reviewed by MacLachlan and Dubovi, 2011). However, the technology supporting these techniques is under constant development and new test platforms, especially those that incorporate multiplexing, are likely to become available for routine diagnostics in the near future (reviewed by MacLachlan and Dubovi, 2011).

Diagnostic assays should be designed and selected to serve a distinctive purpose(s), such as confirming disease-free status, monitoring and eradication, confirmation of a diagnosis, estimating prevalence, and/or identifying infected animals (OIE, 2013). In order to be validated for its purpose, the test should complete three stages of the validation pathway according to OIE standards. The stages include analytical (analytical sensitivity [ASe], specificity [ASp] and repeatability) and diagnostic characteristics (diagnostic sensitivity [DSe], specificity [DSp], and cut-off determination) and reproducibility (OIE, 2013). The diagnostic characteristics of a new diagnostic test (index test) may be evaluated against a perfect reference standard, a so-called ‘gold standard’ (OIE, 2014). However, such a comparison is often difficult to make due to the lack of such a test or the high cost of the test procedure (OIE, 2014), and new tests are typically validated against an imperfect reference standard which may either be conditionally dependent (i.e., measurement of similar analytes, such as antibodies) or independent (OIE, 2014). Different methods of statistical analyses, such as latent class methods (e.g., maximum likelihood and Bayesian methods), can be used to correct the bias resulting from the dependence of these tests and imperfectness of the reference test (OIE, 2014).

2.5.2. CIEP

The mass screening for AMDV infections in farms is based on the detection of anti-AMDV antibodies. The CIEP (additional abbreviations CCIE, CIE, and CCE) method has been used in most mink-producing countries for decades as the standard test for the serodiagnosis of AMDV (reviewed by Porter et al., 1980). From its first application in the 1970s, the test was run with a whole-virus antigen propagated in mink (Cho and Ingram, 1972), but since the 1980s cell-culture (CRFK) derived antigen (mainly AMDV-G strain)

has been preferred (Aasted and Cohn, 1982). The antigen has been commercially available in the USA (manufactured by United Vaccines, since ceased production) and in Denmark by the Antigen Laboratory of the Research Foundation of the Danish Fur Breeders' Association (Danad antigen). In Finland, CIEP was used in Fin Furlab (Vaasa) from 1980 until 2008.

A positive test result is scored as a white precipitin line on an agarose gel (Cho and Ingram, 1972). It is formed by immune complexes when the negatively-charged antigen moves towards the anode and the positively-charged antibodies towards the cathode during electrophoresis (Cho and Ingram, 1972). A positive test result can usually be detected by CIEP from two to three weeks p.i., but may take as long as seven weeks in some cases (Hadlow et al., 1983; Hadlow et al., 1985; Jensen et al., 2014; Farid et al., 2015). Although CIEP has been in use for several decades, thorough validation studies (e.g., according to OIE standards) have not been published. Estimates of the DSe range between 79% and 99% and DSp between 90% and 100% (Wright and Wilkie, 1982; Aasted et al., 1986; Jensen et al., 2011; Dam-Tuxen et al., 2014). Unpublished data of the Animal Health Lab (University of Guelph, Canada) suggest a DSe value of 98%, DSp of 86% to 91%, and repeatability of 98% to 99% (reviewed by Nituch et al., 2011; reviewed by Bowman et al., 2014). Several studies indicate that CIEP gives lower titers than many other test methods and fails to identify low levels of antibodies during early stages of an infection (Crawford et al., 1977; Aasted and Cohn, 1982; Alexandersen and Hau, 1985; Alexandersen et al., 1985a; Aasted et al., 1986; Miroshnichenko et al., 1992; Farid et al., 2015).

2.5.3. Other diagnostic methods

During the late 1960s to late 1980s, numerous non-specific (iodine agglutination test, serum electrophoresis, and glutaraldehyde test) and specific (immunofluorescence, indirect immunofluorescence, complement fixation, viral agglutination, *in situ* hybridization, ELISA, and CIEP) diagnostic tests were developed (reviewed by Gorham et al., 1976; reviewed by Porter et al., 1980; Wright and Wilkie, 1982; Alexandersen et al., 1987; Bloom et al., 1989). The diagnosis of AMDV infection in mink can also be made based on the detection of AMDV DNA by PCR (Jensen et al., 2011), gross and histopathologic lesions (Eklund et al., 1968), and AMDV antigen in tissues by immunohistochemistry (Hammer et al., 2007).

As a first screening test for AMDV, to detect hypergammaglobulinemia in mink with AMDV infection, farmers used the iodine agglutination test (IAT) (reviewed by Porter et al., 1980). For disease control and eradication purposes, this method was problematic as it gave a high proportion of false negative results and identified the positive animals only at a late stage of infection (Cho and Ingram, 1974). In addition to IAT, hypergammaglobulinemia (>20% of total serum protein) caused by AMDV can be identified with serum protein electrophoresis, which has been widely used in research (reviewed by Porter et al., 1980).

For several years, development of the test mainly focused on improving the sensitivity and/or specificity of the CIEP test. Such tests as modified counterelectrophoresis

(Crawford et al., 1977), indirect counter-current electrophoresis (Aasted and Cohn, 1982), counter-current line absorption immunoelectrophoresis (CCLAIE) (Alexandersen et al., 1985a; Aasted et al., 1986), additive CIEP (Uttenthal, 1992), and rocket line immunoelectrophoresis (RIA) (Alexandersen and Hau, 1985) were developed. The most sensitive (both DSe and ASe) of these electrophoretic assays seems to be the CCLAIE (Aasted et al., 1986).

Except for IAT, these tests have not been commonly used for mass screening due to their high cost or large amount of processing time. Instead, they have been applied in confirmatory testing, individual animals and research. In recent years, the development of new assays for the diagnosis of AMDV infection has become more active. New ELISA and PCR-based tests are now available for mass screening and confirmation applications (studies II, III, IV, Jensen et al., 2011; Dam-Tuxen et al., 2014).

2.6. Epidemiology and phylogenetics

2.6.1. Host range, geographic distribution, and prevalence

Clinical signs and lesions are mainly found in farmed and feral/wild American mink in many European and North American countries (Table 3). In farmed mink in Finland, the annual mean seroprevalence of all tested mink ranged between 3% and 60% (1980–2014) (Kangas and Smeds, 1980s, M. Eerola (Fin Furlab), personal communication 21.5.2015). When testing began in 1980, prevalence was as high as 50% to 60% (Kangas and Smeds, 1980s). Nowadays, about 700 000 mink and almost 90% of farms are tested annually and the prevalence is approximately 15% (2014) (M. Eerola [Fin Furlab], personal communication 21.5.2015). Unfortunately, data for other mink-producing countries are scarce.

Other species that may develop AD are the ferret (*Mustela putorius furo*) and striped skunk (*Mephitis mephitis*) (Porter et al., 1982; LaDouceur et al., 2014). Anti-AMDV antibodies and/or AMDV DNA have also been found in other mustelids, such as European mink, weasels, martens, polecats, and otters in Europe, North America, and Japan (for references, see Table 3 and Murakami et al. (2001)). In addition to Mustelidae, other carnivores such as common genets, raccoons, and foxes may become infected (see Table 3 for references). Coyotes (*Canis latrans*), groundhogs (*Marmota monax*), fishers (*Martes pennati*), badgers (*Meles meles*), raccoon dogs (*Nyctereutes procyonoides*), beavers (*Castor canadensis*), muskrats (*Ondatra zibethicus*), and squirrels (*Tamiasciurus hudsonicus*) have been tested for antibodies and/or AMDV DNA, but none have so far been found (Ingram and Cho, 1974; Farid, 2013; Leimann et al., 2015). Although signs of AMDV infection have been detected in several of the species mentioned above, there is yet little evidence that the virus can cause disease or induce clinical symptoms and lesions. However, some indication of subclinical or mild disease exists in European mink (Fournier-Chambrillon et al., 2004).

Experimentally, AMDV has been inoculated into several species. Ferrets, stoats, fishers, American martens (*Martes americana*), raccoon dogs, raccoons, cats, dogs, blue foxes, mice, and rabbits all developed antibodies after inoculation (Kenyon et al., 1978; Porter et al., 1982; Alexandersen et al., 1985b; Oie et al., 1996). Of these species, evidence of viral replication was found in ferrets, raccoons, raccoon dogs and dogs (Porter et al., 1982; Alexandersen et al., 1985b; Oie et al., 1996). Additionally, mice and cats harboured infective virus in their tissues (Alexandersen et al., 1985b). Only ferrets and striped skunks developed histologic lesions resembling AD in mink (Kenyon et al., 1978; Porter et al., 1982; Alexandersen et al., 1985b; Oie et al., 1996).

Anti-AMDV antibodies have also been found in humans (McGuire and Crawford, 1980; Jepsen et al., 2009), often with no associated illness. A recent study found antibodies and AMDV DNA in two mink farmers, one with artheritis and the other with chronic glomerulonephritis (Jepsen et al., 2009). Also, two other suspected cases have been reported earlier (Chapman and Jimenez, 1963; Helmboldt et al., 1965). The diagnosis was based on clinical signs following exposure to mink and comparative pathology, but anti-AMDV antibody or AMDV DNA were not tested as such tests were unavailable at the time (Chapman and Jimenez, 1963; Helmboldt et al., 1965). The persistence of antibodies up to four years after the last contact with mink is a cause for concern (Jepsen et al., 2009), as it may indicate a persistence of the viral infection. However, whether this virus actually has any zoonotic potential needs further proof.

Table 3. *Prevalence and case descriptions of Aleutian mink disease virus infection in different animal species and humans*

Species	Ab%[a]	DNA%[a]	Sampling year	Country	Method	Lesions[b]	Reference
Feral/wild American mink	93 (60)	88 (60)	2009–2011	Canada	CIEP, PCR	ND	(Farid, 2013)
	29 (208)	25 (183)	2005–2009	Canada	CIEP, PCR	ND	(Nituch et al., 2011; Nituch et al., 2012)
	55 (55)	ND	Early 1970s	Canada	CIEP, HP	Histologic lesions typical for AD (6/55)	(Cho and Greenfield, 1978)
	55 (29)	ND	Early 1970s	Canada	ND	ND	(Ingram and Cho, 1974)
	46 (144)	58 (144)	2004–2009	Sweden	ELISA, PCR	Gross lesions typical for AD (6/144)	(Persson et al., 2015)
	3–45 (538)	0-32 (57)	1998–2009	Denmark	CIEP, PCR	ND	(Jensen et al., 2012)
	23 (75)	ND	1996–2002	France	CIEP, CCLAI	Poor physical condition (2/5)	(Fournier-Chambrillon et al., 2004)
	52 (27)	ND	Late 1990s	England	CIEP	ND	(Yamaguchi and

Species	Ab%[a]	DNA%[a]	Sampling year	Country	Method	Lesions[b]	Reference
							Macdonald, 2001)
	ND	15 (27)	2007–2010	Estonia	PCR	ND	(Leimann et al., 2015)
	0 (16)	40 (5)	1997–1999	Spain	CIEP, PCR, HP	Histologic lesions typical for AD (1/5)	(Mañas et al., 2001)
European mink	0 (84)	ND	2004–2005	Spain	CIEP	ND	(Sanchez-Migallon Guzman et al., 2008)
	12 (99)	ND	1996–2002	France	CIEP, CCLAI	Poor physical condition (3/14)	(Fournier-Chambrillon et al., 2004)
	33 (9)	1/1[b]	1997–1999	Spain	CIEP, PCR, HP	No lesions (0/1)	(Mañas et al., 2001)
Polecat	11 (145)	ND	1996–2002	France	CIEP, CCLAI	ND	(Fournier-Chambrillon et al., 2004)
Stone marten	24 (17)	ND	1996–2002	France	CIEP, CCLAI	ND	(Fournier-Chambrillon et al., 2004)
Pine marten	6 (16)	ND	1996–2002	France	CIEP, CCLAI	ND	(Fournier-Chambrillon et al., 2004)
Common genet	4 (68)	ND	1996–2002	France	CIEP, CCLAI	ND	(Fournier-Chambrillon et al., 2004)
Stoat	70 (61)	70 (61)	2009–2011	Canada	CIEP, PCR	ND	(Farid, 2013)
River otter	0 (59)	0 (59)	2011–2012	Canada	CIEP, qPCR	ND	(Bowman et al., 2014)
	18 (11)	18 (11)	2009–2011	Canada	CIEP, PCR	ND	(Farid, 2013)
Ferret	9 (446)	ND	1990–1991	England	CIEP	ND	(Welchman Dde et al., 1993)
	42 (214)	ND	Late 1970s–early 1980s	USA	IF	ND	(Porter et al., 1982)
Striped skunk	ND	7 (27)	2010–2013	USA	PCR, ISH, necropsy, HP	Mild to severe gross and histologic lesions similar to mink AD (7/27)	(LaDouceur et al., 2014)
	18 (22)	14 (22)	1990s	USA	CIEP, PCR	ND	(Oie et al., 1996)
	25 (8)	13 (8)	2009–2011	Canada	CIEP, PCR	ND	(Farid, 2013)

Species	Ab%[a]	DNA%[a]	Sampling year	Country	Method	Lesions[b]	Reference
	41 (347)	32 (40)	2006–2008	Canada	CIEP, PCR	ND	(Nituch et al., 2015)
	65 (196)	ND	Early 1970s	Canada	ND	ND	(Ingram and Cho, 1974)
Raccoon	11 (85)	11 (85)	2009–2011	Canada	CIEP, PCR	ND	(Farid, 2013)
	4 (27)	ND	Early 1970s	Canada	ND	ND	(Ingram and Cho, 1974)
Fox	2 (100)	ND	Early 1970s	Canada	ND	ND	(Ingram and Cho, 1974)
Bobcat	10 (20)	10 (20)	2009–2011	Canada	CIEP, PCR	ND	(Farid, 2013)
Farmed mink	15 (700 000)	ND	2014	Finland	ELISA	ND	Eerola 2015 (pers. comm.)
	48–67 (ND)	ND	2005	China	ND	ND	(reviewed by Li et al., 2012)
	5 (1500–2500)[c]	ND	2001	Denmark	CIEP	ND	(Christensen et al., 2011; Espregueira Themudo et al., 2012)
	80 (5)[c]	ND	2010	Ireland	PCR	ND	(Jahns et al., 2010)
	ND	22 (51)	2007–2010	Estonia	PCR	ND	(Leimann et al., 2015)
	1–5 (3 000 000)	ND	1999–2005	Canada	CIEP	ND	(Farid et al., 2012)
Human	2/2[b]	ND	1990s–2000s	Denmark	CIEP, CCLAI, PCR, autopsy, HP	Arteritis (1/2), glomerulo-nephritis (1/2)	(Jepsen et al., 2009)
	7 (243)	ND	Late 1970s	USA	CIEP, CF	ND	(McGuire and Crawford, 1980)
	0 (18)	ND	1970s	USA	CIEP	ND	(Bloom et al., 1975)

[a] Number of tested individuals in parenthesis.
[b] Number of positive individuals/number of individuals tested.
[c] Number of tested farms.
Abbreviations: Ab, antibody; AD, Aleutian disease; CIEP, counter-current immunoelectrophoresis; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; HP, histopathology; ND, no data; ISH, *in situ* hybridization; IF, immunofluorescence; CCLAI, countercurrent line absorption immunoelectrophoresis; qPCR, real-time quantitative PCR; CF, complement fixation.

2.6.2. Molecular epidemiology and phylogenetics

Since the first report of AD over 60 years ago, details of the AMDV particle have been extensively studied. Initially, research mostly concentrated on a few well-documented AMDV strains of farmed mink identifying genetic markers for pathogenicity and other

biological features by sequence comparison and construction of molecular clones (see Chapter 2.3.1 for references). Originally, these strains were isolated from the pooled organs of many infected mink and have since been propagated in mink or cell culture for several generations (Bloom et al., 1975; Bloom et al., 1980; Hadlow et al., 1983; Alexandersen, 1986; Gottschalck et al., 1994). Thus, these strains do not necessarily represent the viral population in farms.

The first phylogenetic analyses were performed in the 1990s and in the beginning of the 2000s (Gottschalck et al., 1994; Schuierer et al., 1997; Olofsson et al., 1999; Murakami et al., 2001). Sequence data and the applied phylogenetic methods were rather limited, but these first analyses suggested that the AMDV strains isolated from farmed mink can be divided into three major phylogenetic clusters (Olofsson et al., 1999). None of the studies indicated that AMDV strains would group based on the pathogenicity of the strain (Schuierer et al., 1997; Olofsson et al., 1999), country or region, or year of isolation (Schuierer et al., 1997).

Common mechanisms of evolution applied by viruses are mutations, quasispecies formation, recombination, and reassortment (reviewed by MacLachlan and Dubovi, 2011). Data on AMDV evolutionary mechanisms are scarce. The virus seems to have a bias towards amino acid changes (Gottschalck et al., 1994; Schuierer et al., 1997; Olofsson et al., 1999) and it appears to be evolving under slight purifying or positive selection for variation with a d_S/d_N (d_S, substitution rate at silent sites; d_N, substitution rate at non-silent sites) ratio ranging between 0.29 and 1.26 (ratio >1, purifying selection; <1 positive selection) within different areas of the genome (Gottschalck et al., 1994; Schuierer et al., 1997). However, the d_S/d_N ratio is relatively insensitive to the strength of natural selection when sequences from a single population or similar isolates are compared (Kryazhimskiy and Plotkin, 2008). Gottschalck et al. (1994), by including only a few strains, suggested that diversity in AMDV is the product of an ancient history rather than the result of a high mutation rate. They calculated that AMDV strains G and K have separated 700; and G and Utah 1 50 evolutionary years ago (calculated based on canine parvovirus capsid gene, 1.69×10^{-4} /nt substitutions/year). Although many divergent strains exist, they seem to show high genomic stability exhibiting little evolution during six years of observation (Christensen et al., 2011). Recombination has been reported to occur in other parvoviruses (Lukashov and Goudsmit, 2001; Shackelton et al., 2007; Wang et al., 2012; da Costa et al., 2013). As AMDV induces a persistent infection with the potential for superinfections, there are good opportunities for recombination. Thus recombination may also occur in AMDV (Shackelton et al., 2007; Christensen et al., 2011), although further studies are needed.

The phylogenetic and epidemiologic details of AMDV in farmed mink and free-ranging mustelids in Finland have, prior to this study, largely been unknown. Finnish farmed mink strains have only been described in one earlier study; Olofsson (1999) included four strains from farmed Finnish mink in his study of AMDV strains in Sweden. This limited data set showed that the Finnish strains were closely related to Danish K strain and global Utah 1, SL3 and G strains of farmed mink.

3. Aims of the study

The aims of this study were to:

1. Develop AD diagnostics: to construct a new recombinant antigen with which to develop, automate, and validate a serological assay for detecting anti-AMDV antibodies in blood samples of farmed mink and, in addition, to develop a simple blood sampling technique for the assay. (II, III)

2. Evaluate the extent to which AMDV is present in free-ranging mustelids in Finland, estimate the prevalence, and elucidate any factors associated with AMDV infection. (IV)

3. Study the diversity, evolutionary relationships and infer the origin(s) of AMDV strains present in Finnish farmed mink and free-ranging mustelids, compare them to previously reported strains, and deduce possible correlations with their geographical and temporal distribution, transmission routes, pathogenicity, and the extent of their variation. (I, IV)

4. Materials and methods

4.1. Study design and ethics

Studies I and IV utilized cross-sectional designs and phylogenetic methods to study the molecular epidemiology of AMDV in Finnish free-ranging mustelids and/or farmed mink. Additionally, in study IV, analytical epidemiologic tools were used to estimate the prevalence, distribution, and potential risk factors for AMDV infection in Finnish free-ranging mustelids. In studies II and III, cross-sectional designs were used to compare the results of two diagnostic tests (reference test, CIEP; index test, ELISA) in order to evaluate the performance of a newly constructed AMDV VP2 antigen and validate a new automated ELISA test based on this antigen and utilizing a new blood sampling method. The studies were carried out in Finland 2005–2014.

The target population in studies I, II, and III was Finnish farmed mink and the source population was mink from farms sending their AMDV screening samples to Fin Furlab (formerly Fur Animal Feed Laboratory). The study sample was a convenience sample from mink on farms willing to participate in the studies. In study I, the sampled mink (serum, organs) were euthanized due to infertility, which was regarded as a sign of potential AD. Presampling with serological testing was not possible due to breeding season. Blood and serum samples in studies II and III were collected by STKL (T. Hinkkanen) during routine serological screening of AMDV. Although AD is not an OIE-listed disease, the study (III) validating the automated ELISA test was designed following the OIE principles and methods of validation of diagnostic assays for infectious diseases (OIE, 2008). However, an evaluation of ELISA reproducibility could not be performed because the Fin Furlab was the only laboratory using this ELISA test at the time of the study. Results were reported according to the STAndards for the Reporting of Diagnostic accuracy studies (STARD) statement (Bossuyt et al., 2003) with minor modifications (Gardner et al., 2011). More information on the sampling framework, selection of animals and herds, and sampling protocol can be found in study III (Figure 1 and Methods). In studies II and III, the laboratory personnel were aware of the source farms identity. However, tests were either run simultaneously (III) or in a different laboratory (II), thus the personnel were unaware of the results from individual animals.

In study IV, all mustelid species (both wild and feral) occurring throughout the country were targeted. Trapped animals and those that had died of natural causes were the source population. The sample population comprised animals obtained through our research partners (details in 4.2.1). Blood and organ samples were obtained from small game trappers during the hunting season or from carcasses sent to the Finnish Food Safety Authority (Evira) for necropsy.

A license for animal experiment was not required for these studies (Act on the Protection of Animals Used for Scientific or Educational Purposes, 497/2013), as samples were collected either from euthanized, dead, or trapped animals or during routine serological screening. Protected species that are found dead can be delivered to a University for research purposes (Nature Conservation Act, 1096/1996; Hunting Act,

615/1993). All farms voluntarily participated in the studies and all samples were uniquely coded prior to being placed in storage.

4.2. Sampling and sample storage

4.2.1. Samples from farmed mink and free-ranging mustelids

Blood samples were obtained by post-mortem cardiac puncture in blood-collection tubes (I), by toenail cutting in two 80 µl glass capillary tubes (II), or by collecting blood from the heart, wounds, peritoneal or thoracic cavity from a carcass on 2 × 7 cm filter paper (Tervakoski Oy, Tervakoski, Finland) strips (IV). The latter sampling procedure was also applied to Estonian free-ranging mustelids to obtain reference sequences for phylogenetic analysis (for more details see Leimann et al. (2015)). In study III, two blood samples were taken by toenail cutting from each animal, one into an 80 µl glass capillary tube and the other with a newly-developed sampling method; the filter paper blood comb (see more details in 4.8.2). Samples from the liver (I), mesenteric lymph node (I), and spleen (I, IV) were collected into cryo tubes after death. During post-mortem sampling, possible lesions related to AD were recorded (I), although a thorough necropsy was not conducted. Organ samples were stored prior to analysis at -70 °C (I, IV) or -20 °C (IV), blood combs and filter paper strips, after air-drying, at 4 °C (III, IV), room temperature (RT) (IV), or -20 °C (IV) and serum, after centrifugation, at 4 °C (III) or -20 °C (I, II). Detailed information on the sampling is presented in Table 4. The serum was analyzed for anti-AMDV antibodies by CIEP (I, II, III) and ELISA (II) and the filter paper and blood comb samples (III, IV) by ELISA. Background information on the sampling is provided in Table 4.

The samples in study IV were collected through the Natural Resources Institute Finland (Luke) (formerly the Finnish Game and Fisheries Research Institute), the Finnish Food Safety Authority (Evira), and private small game trappers via Luke (formerly the Finnish Forest Research Institute), the Finnish Hunters' Association, and the Finnish Wildlife Agency. The following data from the sampled animals were recorded for study I: farm location and identity, AMDV test prevalence, date, age, sex, genotype, clinical signs, and necropsy findings; study II: farm location and identity, AMDV test prevalence, and date; study III: farm location and identity, AMDV test prevalence, date, age, sex, genotype, vaccination status, and clinical signs; and for study IV: trapping/death location, date, species, gender, and age estimate (juvenile, adult).

Table 4. *Background information on the samples collected for studies I–IV*

Study	Species	N	Samples	Source	Administrative region	AMDV prevalence	Symptoms	Age	Sex	Genotype	Time	Method	Purpose
I	Farmed American mink	17[a]	Organs (spleen, liver, mesenterial lymph node), serum	6 mink farms (A–F)[b]	O, NO	10–91%	Infertility, anorexia, cachexia, loss of fur, epistaxis	1–3 y	1 ♂; 16 ♀	15 non-Aleutian; 2 Aleutian	Spring 2005	NS1-PCR, CIEP	Molecular epidemiology
II	Farmed American mink	525	Serum	3 mink farms; 1, 2, and 3	O	1: 0%; 2: >80%; 3: >80%	ND	ND	ND	ND	Summer 2007	CIEP, manual ELISA	Construction and evaluation of a new antigen, development of an ELISA test
III	Farmed American mink	761[c]	Serum, blood	2 mink farms; 1 and 2.	CO	1: 81%; 2: 2%	Farm 1: history of clinical AD; farm 2: no history of clinical AD	1–4 y	46 ♂; 715 ♀	Non-Aleutian	Spring 2008	CIEP, automated ELISA	Automation and validation of the ELISA test, development of a new blood sampling method
IV	Feral American mink, European pine marten, European badger, Eurasian otter, European polecat, least weasel, wolverine, stoat	308	Blood, spleen[d]	Wild	17/19 regions in Finland	ND	ND	Juvenile-adult[e]	195 ♂; 110 ♀[f]	ND	2006–2014	NS1-qPCR, manual ELISA	Molecular and analytical epidemiology

[a] 2–3 samples/farm.
[b] Farms D, E, and F had recently encountered severe AD outbreaks just after regaining AMDV-free status.
[c] Farm 1, 361 samples; farm 2, 400 samples.
[d] Spleen samples were obtained from 263 animals.
[e] Age was recorded from 186 animals.
[f] Sex was recorded from 305 animals.
Abbreviations: AMDV, Aleutian mink disease virus; NS1, non-structural protein 1; PCR, polymerase chain reaction; CIEP, counter-current immunoelectrophoresis; ELISA, enzyme-linked immunosorbent assay; qPCR, real-time quantitative PCR; O, Ostrobothnia; NO, North Ostrobothnia; CO, Central Ostrobothnia; ND, no data.

4.2.2. Sample size calculations (III, IV)

The sample size to estimate diagnostic sensitivity (DSe) and specificity (DSp) in the automated ELISA validation study (III), was calculated with the following formula: $n = \left(\frac{z_{1-\frac{\alpha}{2}}}{e}\right)^2 \theta(1-\theta)$, where $z_{1-\alpha/2} = 1.96$, $\alpha = 0.05$, θ is the estimate of DSe or DSp, and e is the desired error margin on the estimate (Greiner and Gardner, 2000). Conservative estimates of 90% of ELISA DSe and DSp (previous estimates have been at least 97% for both (II, Andersson and Wallgren, 2013) were used with 5% allowable error, 95% confidence levels, and with assumption that each population would have 0% or 100% test prevalence. The appropriate sample size was 138. However, approximately 400 samples per population were targeted to account for test prevalence information from 2007 on the two study farms (Farm 1: high prevalence 81%; and farm 2: low prevalence 2%).

The targeted sample sizes in the free-ranging mustelid study (IV) were 5–60 individuals to detect disease in each species or area and 385 to estimate prevalence in the whole mustelid population. Sample sizes were calculated with EpiTools (Sergeant, 2013), and following parameter values were used for detecting disease: unknown population, 5–50% prevalence, 99% test sensitivity, 95% population sensitivity; and for estimating prevalence: infinite population, 50% prevalence, 95% confidence level, and 5% error.

4.3. Nucleic acid extraction (I, IV)

DNA was isolated from 10 mg (IV) or 100 mg (I) of organ (I, IV) or blood (IV) sample. Organ samples were homogenized with a mortar and pestle (I) or lysed in a tissue lysis buffer (IV). Blood from filter paper (1 cm^2) was eluted in 200 µl of phosphate buffered saline (PBS) (IV). DNA was extracted with TriPure (Roche, Indianapolis, USA) (I) or Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) (IV). The DNA concentration was quantified by UV spectroscopy (NanoDrop 1000, Thermo Fisher Scientific, Pittsburgh, PA, USA), and the DNA was stored at -20 °C.

In study I, DNA was extracted from all sampled animals and organs. In study IV, DNA was extracted from blood samples of 92/308 animals [all initially seropositive (n = 43), samples with S/P just below the cut-off (0.150–0.200, n = 3), and equal number (selected with simple random sampling without replacement) of seronegative samples (n = 46)] (more data on the cut-offs in 4.8.2). Spleen (IV), if available, was used only if the blood of a seropositive animal was negative in real-time quantitative PCR (qPCR).

4.4. Amplification and detection of AMDV DNA by PCR (I, II, IV)

For the molecular epidemiology study (I) in Finnish farmed mink, a part of AMDV *NS1* gene was amplified from the extracted DNA via a semi-nested PCR using previously

reported primers (Table 5). The reaction mixtures are shown in Table 6 and PCR conditions in Table 7.

To construct the new antigen (II) to be used in serological assays, the complete AMDV *VP2* gene was amplified prior to cloning with newly-designed primers from a Finnish farmed mink strain (FIN05/C8) described in study I.

For detecting AMDV or closely-related amdoparvovirus DNA in free-ranging mustelids (IV) (as these potentially carry divergent strains), primers based on the recently described gray fox amdovirus (Li et al., 2011) and a qPCR protocol were developed. Fluorescence was measured at the end of the annealing step. A further segment to determine the dissociation curve of the PCR products was performed according to the manufacturer's protocol. Melting curve analysis was used to control the specificity of the products. Two runs were completed per sample and were regarded PCR positive when ≥10 copies were amplified in both runs. If qPCR and ELISA gave discordant results, DNA isolation (from spleen if available), qPCR, and ELISA were repeated and the potential qPCR product was sequenced and verified to be AMDV DNA by a BLAST search (BLAST, 2014).

A standard curve for viral quantitation (IV) for the qPCR was created by amplifying a part of the *NS1* gene harboring the target sequence of the qPCR. After purification, the DNA concentration was quantified by UV spectroscopy (NanoDrop 1000, Thermo Scientific, Pittsburgh, PA, USA) and the copy number was calculated (Thermo Fisher Scientific, 2014). A standard curve was then created with seven ten-fold dilutions of the product ranging from $1{:}10^0$ to $1{:}10^6$. A threshold fluorescence level was calculated with MxPro qPCR software (Agilent Technologies, Santa Clara, CA, USA) using the individual threshold option. The correlation coefficient was 0.966, PCR amplification efficiency 81.6%, and detection limit ten copies.

PCR reagents were supplied by Fermentas (Burlington, Canada) (I, II), Thermo Scientific (Pittsburgh, PA, USA) (IV), and Agilent Technologies (Santa Clara, CA, USA) (IV). The traditional PCRs were performed using PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, USA) (I, II) and the qPCR by using Stratagene Mx3005P thermocycler (Agilent Technologies, Santa Clara, CA, USA) (IV). The PCR products were stained with ethidium bromide (I, II) or GelRed (Biotium, Inc., Hayward, CA, USA) (IV), run on 1% agarose gel (SeaKem LE Agarose, Lonza Group Ltd., Basel Switzerland or NuSieve Agarose, Cambrex BioScience Rockland Inc., Rockland, ME, USA) in tris(hydroxymethyl)aminomethane-borate-ethylenediaminetetraacetic acid (Tris-borate-EDTA, TBE) (I, II) or Tris-acetate-EDTA (TAE) (IV) buffer, and after 30–45 min of electrophoresis at 80–85 V, were visualized under UV light.

From each animal, one PCR product was purified from the reaction mixture with QIAquick PCR Purification kit (Qiagen, Hilden, Germany) (I) or treated with exonuclease I and thermosensitive alkaline phosphatase (Thermo Scientific, Pittsburgh, PA, USA) (IV). The products that were used for cloning (II) or creating a standard curve (IV), were purified from the gel with QIAquick gel extraction kit (Qiagen, Hilden, Germany).

Contamination was avoided by using sterile filter-tips, protective clothing, detergent disinfectants, ultraviolet light, and unidirectional sample movement and negative controls were included in every PCR. Sample preparation, DNA extraction, PCR mix preparation, template pipetting, amplification, product purification, serological assays, and cloning

were performed in separate laboratories and two different facilities (the Faculty of Veterinary Medicine and the Faculty of Medicine, University of Helsinki).

Table 5. *PCR primers used for the amplification and detection of AMDV DNA*

Target gene	Size, nt	Position[a]	Forward primer	Reverse primer	Reference
***NS1* partial (I)**	390	563–952	5′-TTA GCT TTT GAC TCT ATT GAA GAG A-3′[b] 5′-AAA ACT CAG CAA TTT CAT ATT CAC-3′[c]	5′-TCG TTC TTT GTT AGT TAG GTT GTC-3′	(Olofsson et al., 1999)
***VP2* complete (II)**	1944	2406–4349	5′-TTT GGA TCC AAT AGA GGA AAT GGA TTC TGC TG-3′[d]	5′-TTT GAC GTC TTA GTA GAT ATA TTT GAT AGT GCT TCT TCC-3′[e]	II
***NS1* partial (IV)**	641	1662–2302	5′-AAG ACT TTA AAG CCA TTA CTG GA-3′	5′-CTT TAG TTC CTC AGC ACT ATC C-3′	IV
***NS1* long (IV)[f]**	1725	578–2302	5′-CAT ATT CAC TGT TGC TTA GGT TA-3′	5′-CTT TAG TTC CTC AGC ACT ATC C-3′	IV; (Jensen et al., 2011)

[a] Nucleotide position corresponding to the compete sequence of the AMDV-G strain (GenBank accession nos. M20036 (I), NC_001662 (II), or JN040434 (IV)).
[b] 1st round.
[c] 2nd round.
[d] *Bam*HI digestion site underlined.
[e] *Pst*I digestion site underlined.
[f] Used for creating the standard curve for qPCR.
Abbreviations: PCR, polymerase chain reaction; AMDV, Aleutian mink disease virus; NS1, non-structural protein 1; VP2, structural protein 2; qPCR, real-time quantitative PCR.

Table 6. *PCR reaction mixtures used in the amplification and detection of AMDV DNA*

Component	Study I, *NS1* partial	Study II, *VP2* complete	Study IV, *NS1* partial	Study IV, *NS1* long
PCR Buffer with $(NH_4)_2SO_4$	1×	1×		1×
Maxima SYBR Green Master Mix/ROX			1×	
dNTP mix	0.2 mM	0.2 mM		0.2 mM
$MgCl_2$	1.5 mM	1.5 mM		2 mM
Forward and reverse primer	0.5 µM	0.5 µM	0.6 µM	1 µM
***Taq* polymerase**	2.5 U	2.5 U		2.5 U
***Taq* Extender**				2.5 U
Template DNA	1st round, 5 µl; 2nd round, 2 µl	5 µl	5 µl	10 µl
Molecular biology grade water	To 100 µl	To 100 µl	To 25 µl	To 100 µl

Abbreviations: PCR, polymerase chain reaction; AMDV, Aleutian mink disease virus; NS1, non-structural protein 1; VP2, structural protein 2; dNTP, deoxynucleoside triphosphate.

Table 7. *PCR cycling conditions used in the amplification and detection of AMDV DNA*

Step	Study I, *NS1* partial	Study II, *VP2* complete	Study IV, *NS1* partial	Study IV, *NS1* long
Initial denaturation	95 °C 10 min	95 °C 10 min	95 °C 10 min	94 °C 5 min
Cycles	1st round, 34; 2nd round, 29	1st round, 5; 2nd round, 30	40	40
Denaturation	95 °C 30 s	95 °C 30 s	95 °C 30 s	95 °C 30 s
Annealing	1st round, 56 °C; 2nd round, 54 °C 30 s	1st round, 54 °C; 2nd round, 59 °C 30 s	55 °C 1 min	55 °C 45 s
Extension	72 °C 1 min	72 °C 1st round, 3 min; 2nd round 2.5 min	72 °C 1 min	72 °C 1.5 min
Final Extension	72 °C 10 min	72 °C 10 min		72 °C 7 min

Abbreviations: PCR, polymerase chain reaction; AMDV, Aleutian mink disease virus; NS1, non-structural protein 1; VP2, structural protein 2.

4.5. Sequencing (I, II, IV)

The purified PCR products (I, IV) and VP2-plasmid constructs (II) were sequenced with forward and reverse primers of the second round amplification (I), twice with the reverse primer (IV), or with commercial primers (M13, T7, and SP6) (II). Sequencing was performed with Biosystems Dye Terminator (v.3.1) sequencing kit and 3100 capillary sequence analyzer by the Sequencing Core Facility (Department of Virology, Faculty of Medicine, University of Helsinki). From each PCR product, two or three sequences were aligned and a consensus sequence was generated.

For the phylogenetic analyses in study I, one PCR-positive organ (usually spleen) per animal was sequenced (I). In study IV, up to three PCR-positive samples per region were sequenced favoring other species than mink (a total of 20/34 positives were sequenced).

4.6. Sequence analysis (I, IV)

The acquired nucleotide sequences were assembled and translated with BioEdit 7.0.5 (Hall, 1999) (available at www.mbio.ncsu.edu/bioedit/bioedit.html) (I, IV) and GeneDoc 2.6.0.2 (Nicholas and Nicholas, 1997) (I). Available reference sequences were collected from the GenBank database (GenBank, 2008; 2014). In study IV, additional reference sequences were obtained from Estonian free-ranging mustelids (Leimann et al., 2015), Finnish farmed mink acquired through diagnostic samples sent to the Department of Virology, and from study I. For more details of the sequences used see Appendix I. A species- and strain-demarcation criterion is currently lacking for amdoparvoviruses (Tijssen et al., 2011). In other genera of the *Parvovirinae*, strains that are <95% identical (i.e., >5% divergent) for the *NS* gene sequence are regarded as different species (Tijssen et al., 2011). Here, each unique sequence obtained was defined as a strain.

Identity matrices of nucleotide and amino acid sequences were constructed with BioEdit 7.0.5 (Hall, 1999). The rates of synonymous (d_S) and non-synonymous (d_N) nucleotide substitutions were calculated with SNAP (Korber, 2000) (available at www.hiv.lanl.gov) using the Nei and Gojobori (1986) method (I).

A multiple sequence alignment was assembled with ClustalW 1.83 (Thompson et al., 1994) using default values. The phylogenetic analyses of study I were carried out with MEGA 3.1 (Kumar et al., 2004) using the maximum parsimony (MP) method with heuristic close-neighbor interchange search and PAUP* 4.0 (Swofford, 2003) with the maximum likelihood (ML) method. An appropriate model for ML, general time reversible + invariant sites + gamma (GTR + I + G), was determined with the Modeltest 3.7 (Posada and Crandall, 1998) run in PAUP* 4.0. In study IV, the ML method with MEGA5 (Tamura et al., 2011) and Bayesian method with BEAST 2 (Bouckaert et al., 2014) and a GTR model were used. Bootstrap resampling (1 000 pseudoreplicates) was performed to evaluate support for phylogenetic trees. Human parvovirus B19 (GenBank accession number (acc. no.) AY903437) and porcine parvovirus (acc. no. DQ499631) (I), or gray fox amdovirus (acc. no. JN202450) (IV) were used as outgroups.

Recombination (I) between different groups of strains was analyzed with SplitsTree4 (Huson and Bryant, 2006) using the HKY85 method (Hasegawa et al., 1985). Rate homogeneity (i.e., a molecular clock) (I) was tested with TreePuzzle (Schmidt et al., 2002) using ML (GTR model) and 25 000 puzzling steps. Rate heterogeneity with a uniform rate of nucleotide substitutions, gamma distribution with eight rate categories, and mixed model (1 invariable + 8 gamma rates) was used.

4.7. Recombinant VP2 antigen (II)

4.7.1. Construction and expression

The purified VP2 PCR product was ligated to pGEM-T plasmid (Promega, Madison, WI, USA) and transformed into *Escherichia coli* JM109 competent cells (Promega, Madison, WI, USA) according to the manufacturer's instructions. Bacteria were grown in Luria broth (LB) for 1 h in a shaker. For blue/white screening, the bacteria were plated on LB agar plates containing ampicillin (250 µg/ml), isopropyl-β-D-thiogalactopyranoside, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (IPTG and X-Gal) and incubated overnight (o/n) at 37 °C. White bacterial colonies were suspended in LB containing ampicillin (100 µg/ml) and incubated o/n at 37 °C. Plasmid DNA containing the VP2 insert was isolated with QIAprep Spin miniprep kit (Qiagen, Hilden, Germany) from the bacterial culture and digested with restriction enzymes *Bam*HI and *Pst*I (New England Biolabs, Ipswich, MA, USA) along with the baculovirus transfer plasmid pAcYML1 (provided by Johan Peränen, Institute of Biotechnology, University of Helsinki; the plasmid was modified from Matsuura et al. (1987)). The VP2 insert and pAcYML1 plasmid were run on an agarose gel, purified (QIAquick gel extraction kit, Qiagen, Hilden, Germany), ligated (T4 DNA ligase, New England Biolabs, Ipswich, MA, USA), transformed into *Escherichia coli* and grown as described above. Plasmid constructs were isolated from the bacterial suspension with EndoFree plasmid maxikit (Qiagen, Hilden, Germany).

The purified plasmid DNA (2 µg) and baculovirus DNA (250 ng) (BaculoGold, Pharmingen, San Diego, CA, USA) were combined in transfection buffer containing FuGENE (Roche Diagnostics, Indianapolis, IN, USA) and Sf-900 medium (Gibco, Paisley, United Kingdom). The transfection medium was applied to *Spodoptera frugiperda* 9 (Sf9) cells (ATCC CRL-1711; American Type Culture Collection, Rockville, MD, USA) (3×10^6 cells per 25-cm^2 flask) for 2 h at RT and 1 h at 27 °C. The transfection medium was discarded and the cells were grown in fresh Sf-900 medium supplemented with 10% fetal calf serum (Gibco, Paisley, United Kingdom), 1 × glutamine-penicillin-streptomycin (Haartbio, Helsinki, Finland), and 0.25 mg/ml Fungizone (Bristol-Myers Squibb, Rueil-Malmaison, France) at 27 °C for four days. The cells and supernatant were separated by centrifugation (1 300 g for 5 min) and the cell paste was washed four times with phosphate-buffered saline (PBS) containing protease inhibitor (Roche Diagnostics, Indianapolis, IN). Subsequently, the cell paste was stored at -70 °C until processed to be used as an antigen and the supernatant was stored at 4 °C and used for subsequent infections.

Once verified that the recombinant baculovirus produced antigen detectable by anti-AMDV antibodies and after reaching a multiplicity of infection (MOI) of >1 after several subsequent passages, 1 ml of the supernatant was used for Sf9 cells in 25-cm^2, 2 ml for 50-cm^2, and 4 ml for 75-cm^2 flasks. The cells were incubated for 48 h to 72 h, until a cytopathic effect (CPE) was seen.

4.7.2. Extraction and purification

A previously described method (Sico et al., 2002) with tris buffer (20 mM, pH 9) and heating was used to extract and purify the recombinant VP2 protein from the Sf9 cell paste. The method was applied with the following modifications: the cell paste was sonicated with 400 W for 4 × 5 sec pulses in 2 ml tubes, heated at 50 °C for 20 min, and centrifuged at 16 000 g for 30 min. The supernatant was then used as an antigen to establish recombinant VP2 CIEP and ELISA. To be used in CIEP, the recombinant antigen was first diluted 1:16 in PBS containing 0.05% bovine serum albumin (BSA) (Sigma-Aldrich, Steinheim, Germany) and 50 g/ml bromophenol blue (Merck Chemicals, Darmstadt, Germany).

4.8. Serological methods

4.8.1. CIEP (I, II, III)

Anti-AMDV antibodies were measured from the serum samples by CIEP in Fin Furlab (formerly Fur Animal Feed Laboratory) (Vaasa, Finland). CIEP was performed using a commercially available (I, II, III) (Danad, Antigen Laboratory of the Research Foundation of the Danish Fur Breeders' Association, Glostrup, Denmark) or newly developed recombinant VP2 (II) antigen. Counter-current immunoelectrophoresis was performed according to the manufacturer's (Danad) instructions and results were visually inspected and interpreted by Majvor Eerola, Pia Söderback, and Mervi Houtsanen (Fin Furlab, Vaasa, Finland), each with several years of experience using the assay. Results were recorded as positive, negative, or unclear negative/positive.

In studies II and III, CIEP was used as a reference standard (although an imperfect one) as it was the only test that was commercially available and it had been used for decades for large-scale screening of anti-AMDV antibodies in mink. In study II, CIEP with the commercial antigen was tested against CIEP (n = 209) and manual ELISA (n = 316) utilizing the recombinant VP2 antigen. In study III, the performance of CIEP with the commercial antigen was compared to the automated AMDV VP2 ELISA using blood comb sampling.

4.8.2. ELISA (II, III, IV)

A simple and fast indirect ELISA method was developed for automated screening for anti-AMDV antibodies in serum and filter paper blood samples. Optimal dilutions of the reagents were verified with chessboard titrations. In studies II and IV, the ELISA test was run manually, but in study III, the test was automated. The ELISA procedure is depicted in Figure 2. All ELISA procedures were done on either Nunc (II, III) (Finnzymes, Espoo, Finland) or Costar (IV) (Corning Inc., Corning, NY, USA) 96-well immunoplates, using

100-µl reagent volumes. A dilution buffer consisting of PBS with 0.05% Tween 20 and 0.5% BSA was used in all serum and reagent dilutions (except the antigen).

In the validation study (III), the ELISA method was applied with some modifications to suit for automation and robotics (see below). The blood combs, a new blood sampling method utilized in this study, were placed on the ELISA plate containing 100 µl of dilution buffer and soaked for 2 min. Thereafter the blood combs were discarded and the plate was incubated at RT for 1 h.

In the free-ranging mustelid study (IV), the ELISA test was performed with the following modifications. One cm^2 of filter paper saturated with blood was eluted overnight at 4 °C in one ml of PBS after which the filter paper was discarded. The eluted sample was diluted 1:10 in dilution buffer and 100 µl was pipetted in duplicate to an ELISA plate. The ELISA procedure was optimized for the new sample material with chessboard titrations with the antigen and conjugate.

Results were expressed either as net OD values (mean OD for two blank wells was subtracted from the raw OD value) (II, III) or as sample to positive control (S/P) ratio [(mean sample optical density [OD] – mean blank well OD) ÷ (mean positive control OD – mean blank well OD)] (IV).

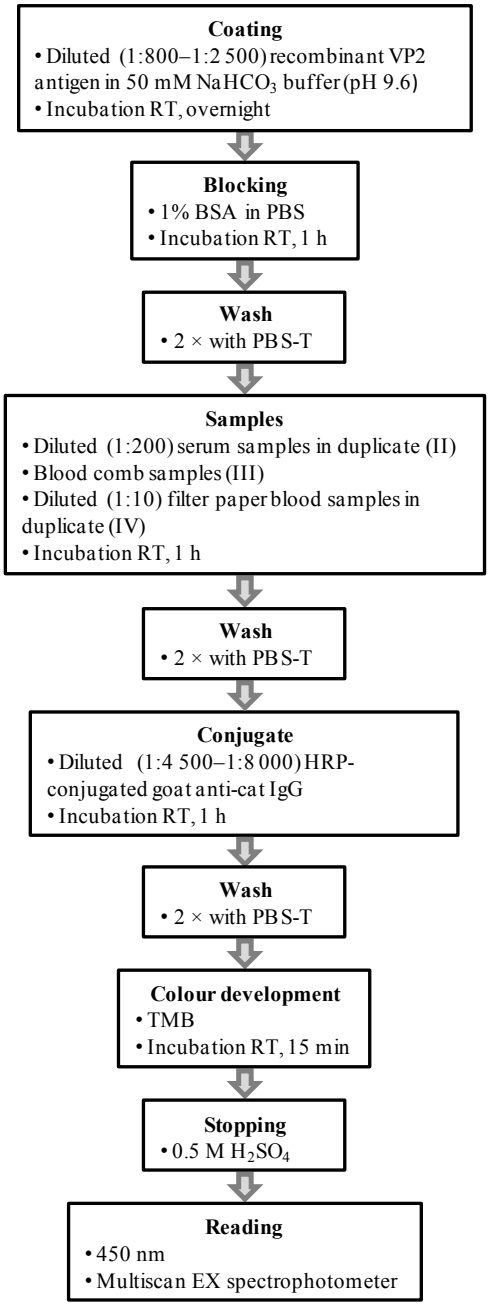

Figure 2. *The ELISA procedure used for AMDV VP2 ELISAs in studies II, III, and IV.*
Abbreviations: VP2, structural protein 2; RT, room temperature; BSA, bovine serum albumin; PBS-T, PBS containing 0.05% Tween 20; HRP, horseradish peroxidase; IgG, immunoglobulin G; TMB, 3,3',5,5'-tetramethyl benzidine; ELISA, enzyme-linked immunosorbent assay; AMDV, Aleutian mink disease virus.

Cut-off (II, III, IV)

Cut-offs for the ELISA tests were determined either by counting the mean OD value of the CIEP-negative samples plus one standard deviation (SD) (II) or by using receiver operating characteristics (ROC) curve analysis with EpiTools (Sergeant, 2013) (III, IV) and choosing a cut-off that gave maximal values for both DSe and DSp when compared to CIEP (III) or qPCR (IV) results. The cut-off in study II was 0.255, III 0.248, and IV 0.292. In study IV, a preliminary cut-off was first set at S/P 0.2 by visual inspection of the histogram of the S/P values of all samples and a proportion of these (see Chapter 4.3) were selected for qPCR.

Blood combs (III)

A new blood sampling method, the so-called 'blood comb', manufactured from filter paper (Tervakoski Oy, Tervakoski, Finland) was developed for collecting the blood samples from farmed mink (see Figure 5 in study III). Twelve samples can be taken with one comb that corresponds to a single row of the ELISA plate. To block the comb from absorbing the liquid in the wells, the upper part was coated with a thin lacquer. A specific device was manufactured by CNC-Tekniikka Oy (Vaasa, Finland) for introducing the blood combs to the ELISA plate (see Figure 6 in study III).

Automation (III)

The automated ELISA system was built with the following devices supplied by Thermo Labsystems (Vantaa, Finland): Cat X robot, Wellwash AC washer, three Multidrop 384 dispensers, and Multiscan Ascent EX spectrophotometer. The system was programmed using Polara 2.3 software. The automated steps were as follows: coating with prediluted antigen, dispensing the dilution buffer for blood comb samples, prediluted conjugate, 3,3',5,5'-tetramethylbenzidine (TMB), and stopping solution, washing, reading, raw data capture, categorizing, presenting, and storing the results.

4.8.3. Western blot (II)

The identity and antigenicity of the recombinant VP2 protein was confirmed with sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis and Western blot analysis. The Sf9 cell pellet infected with the recombinant baculovirus (1:100), non-infected Sf9 cell pellet (1:100), and the purified recombinant VP2 antigen (1:10) were diluted in reducing Laemmli sample buffer and heated for 5 min at 95 °C (Laemmli, 1970). The samples were electrophoresed through a 10% SDS-PAGE gel. The proteins were visualized with Coomassie blue staining and transferred from the gel to a nitrocellulose membrane. TE (0.5 M Tris, 1.5 M sodium chloride, 0.05 M EDTA) buffer containing 0.05% Tween 20 and 1% non-fat milk powder was used for blocking the

membrane and serum and conjugate dilutions. The membrane was blocked o/n at 4 °C and incubated with pooled sera from AMDV-infected and non-infected mink (1:300 dilution) for 1 h at RT. The membrane was washed with TE buffer containing 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated goat anti-cat IgG (1:5 000 dilution; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at RT. After the second wash, the antigen-antibody reaction was visualized with 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich, Steinheim, Germany) staining.

4.9. Control samples

4.9.1. CIEP (I, II, III)

Positive and negative controls provided by the CIEP antigen manufacturer (Antigen Laboratory of the Research Foundation of the Danish Fur Breeders' Association, Glostrup, Denmark) were included on each gel.

4.9.2. PCR (I, II, IV)

Control material for AMDV DNA detection included DNA extracted from naturally infected and AMDV-free farmed mink (I, II) and free-ranging mustelids (IV) obtained from studies I and IV. Samples were verified as infected or AMDV-free with CIEP, Western blot, and/or ELISA (II) and VP2- (Oie et al., 1996) and/or NS1-PCR (Olofsson et al., 1999). Control DNA was stored at -20 °C in small aliquots. Positive and negative controls were included in each PCR run.

4.9.3. ELISA (II, III, IV)

Control sera for the detection of anti-AMDV antibodies was obtained from naturally infected and AMDV-free farmed mink (II, III) or free-ranging mustelids (IV) obtained from studies I and IV. The samples were verified as infected or AMDV-free with CIEP and/or Western blot and VP2- (Oie et al., 1996) and/or NS1-PCR (Olofsson et al., 1999). Control sera were stored at -20 °C in small aliquots. Duplicates of positive, low-positive (II, IV), a negative control serum and blank wells (containing all reagents except blood or serum) were included on each plate. The following dilutions were used: negative 1:200, low positive 1:40 000, and positive 1:200 (II); negative 1:4 and positive 1:300 (III); negative 1:10, low positive 1:80, and positive 1:120 (IV).

4.10. Electron microscopy (II)

The Sf9 cell pellet infected with recombinant baculovirus was stained with 2% tungstophosphoric acid (pH 6.0) and studied with a Jeol JEM-100 CXII electron microscope (Advanced Microscopy Unit, Faculty of Medicine, University of Helsinki).

4.11. Statistical methods (II, III, IV)

Performance of the recombinant VP2 antigen and ELISA test (II) were assessed against the commercially-available CIEP test and validation of the automated ELISA test (III) was performed using several statistical methods (Table 8). A box-and-whisker plot of the ELISA results (III) was generated with EpiTools (Sergeant, 2013).

In the free-ranging mustelid study (IV), statistical analyses were performed to evaluate the factors associated with infection. The outcome variable was infected (1) or AMDV-free (0) and the animal was regarded infected if either ELISA or qPCR test or both were positive. The reason is that the animal might still be seropositive although it has cleared the virus, and the virus might be present but the antibodies are not yet detectable. Species, age (juvenile and adult), sex, year, and location (municipality and administrative region)[6] were independent variables. Due to a limited number of observations in some of the independent variables, they were regrouped as follows: species were divided into mink, badger, and other species; years when the samples were collected were separated into two categories, 2006–2009 and 2010–2014; municipalities were grouped into two categories, those that had no mink farms and those that had mink farms in the area during the 21st century; regions were handled as three different variables, region with two categories (the presence of no mink farms and mink farms in the region), with three categories (no farms, ≤15 farms, and >15 farms in the region), and with 17 categories (total number of sampled regions). All location variables were analysed with separate models. Table 8 presents more data on the statistical methods used in each study.

[6] *Finland is divided into 320 municipalities and 19 administrative regions.*

Table 8. *Statistical methods used in studies II, III, and IV*

Study	Purpose	Method/Program	Details	References
II	Diagnostic Se and Sp with 95% CI	Se=a/(a+c); Sp=d/(b+d)[a]; confidence limits for a proportion (Jeffreys interval), EpiTools.		(Brown et al., 2001; Sergeant, 2013)
	Overall proportion agreement with 95% CI	(a+d)/(a+b+c+d)[a]; confidence limits for a proportion (Jeffreys interval), EpiTools.		(Brown et al., 2001; Sergeant, 2013)
III	Analytical Se	End-point dilutions	Six 10-fold dilutions ranging from 1:10 to $1:10^6$ (two replicates/dilution) of the serum[b] were tested in both ELISA and CIEP. A dilution with positive results on both replicates was set as the detection limit.	
	Analytical Sp	Cross-reactivity against other pathogens present in mink	Animals vaccinated against mink enteritis virus, *Pseudomonas aeruginosa*, and *Clostridium botulinum* were tested.	
		Ability to identify different AMDV strains	Samples from different countries, regions and mustelid species (I, IV, and Knuuttila, 2014 unpublished observations) were tested.	
	Overall, negative, and positive proportion agreement with 95% CI	Compare two tests and confidence limits for a proportion (Jeffreys interval), EpiTools		(Brown et al., 2001; Sergeant, 2013)
	Kappa	Compare two tests, EpiTools		(Sergeant, 2013)
	Diagnostic Se, Sp, and prevalences	2-test 2-population Bayesian model, WinBUGS 1.4.3	Conditional dependence, informative beta priors (study III, Table 4), non-informative (beta (1,1)) priors	(Lunn et al., 2000; Georgiadis et al., 2003; Branscum et al., 2005)
	Model convergence	Visual inspection of trace plots and running 3 chains from dispersed initial values, WinBUGS 1.4.3		(Lunn et al., 2000)
	Probability that ELISA DSe and DSp greater than CIEP DSe and DSp	STEP function, WinBUGS 1.4.3		(Lunn et al., 2000)

Study	Purpose	Method/Program	Details	References
	Predictive values	PPV: the probability that an animal with a positive test result is truly positive; NPV: the probability that an animal with a negative test result is truly negative, WinBUGS 1.4.3.	Informative beta priors on CIEP accuracy and prevalences (study III, Table 4)	(Lunn et al., 2000)
	Within-run variability	Variation in results of the replicates within the same run. CV% = SD ÷ mean × 100%.	Four replicates of each serum[b] on a single plate	
	Between-run variability	Variation in results of the replicates between different runs. CV% = SD ÷ mean × 100%.	Duplicates of each serum[b] from 20 runs on 5 different days	
	Within-serial repeatability variability	Variation in results of the replicates between different reagent batches. CV% = SD ÷ mean × 100%.	Duplicates of each serum[b] against 3 different batches of antigen	
IV	Prevalences with 95% CI	Crosstabulation, SPSS Statistics version 21 and 22; confidence limits for a proportion (Jeffreys interval), EpiTools.		IBM Corp., Armonk, NY, USA; (Brown et al., 2001; Sergeant, 2013)
	Pairwise associations	Fischer's exact test and single variable logistic regressions, SPSS Statistics version 21 and 22	Variables with P ≤0.2 included in multivariable logistic regression analysis and with Wald's P <0.05 in the final model	IBM Corp., Armonk, NY, USA
	Correlation	Spearman's test, SPSS Statistics version 21 and 22	Variables with significant (P <0.05 and correlation coefficient >±0.2) correlation were not used in the same model. Correlation was found between species and 2 of the region variables (all regions and regions with farms vs. no farms).	IBM Corp., Armonk, NY, USA
	Confounders	Causal diagram. Verifying the impact on the final model, SPSS Statistics version 21 and 22.		IBM Corp., Armonk, NY, USA
	Risk factors	Multivariable logistic regression, SPSS Statistics version 21 and 22		IBM Corp., Armonk, NY, USA
	One-level interactions	Verifying the impact on the final model, SPSS Statistics version 21 and 22		IBM Corp., Armonk, NY, USA
	Goodness of fit	Omnibus tests of model coefficients, Nagelkerke's R^2, Hosmer and Lemeshow test, AUC. SPSS Statistics version 21 and 22.	Good fit: Omnibus test, small P value; Nagelkerke's R^2, value close to 100%; Hosmer and Lemeshow test, large P value; AUC, value close to 1.	IBM Corp., Armonk, NY, USA

Study	Purpose	Method/Program	Details	References
	Outliers	Studentized residuals, SPSS Statistics version 21 and 22.	Residuals >2.5 were verified	IBM Corp., Armonk, NY, USA

[a] a, true positive; b, false positive; c, false negative; d, true negative.
[b] Positive control serum was used for the dilutions.
[c] One negative and one low-positive mink serum was used, diluted and handled as the control sera (4.8.3).
Abbreviations: Se, sensitivity; Sp, specificity; DSe, diagnostic sensitivity; DSp, diagnostic specificity; PPV, positive predictive value; NPV, negative predictive value; CV, coefficient of variation; SD, standard deviation; AUC, area under the curve; CI, confidence interval.

5. Results

5.1. Development of a new diagnostic test

5.1.1. Expression and evaluation of the recombinant VP2 protein (II)

To develop a new antigen for the diagnostic test, the *VP2* gene of a Finnish AMDV strain (FIN05/C8) was transferred to a baculovirus and the protein was expressed in Sf9 insect cells and purified. The *VP2* gene was used as the protein forms empty capsids, or virus-like particles (VLPs), that offer an optimal conformation for antigenic recognition. The presence and suitability of the recombinant VP2 antigen for serological assay was assessed with sequencing, electron microscopy, SDS-PAGE and Coomassie blue staining, Western blot, and by comparing the results of CIEP and ELISA performed with the recombinant antigen with the results of CIEP performed with the commercial antigen (consisting virus grown in cell culture).

The sequence and reading frame of the insert were confirmed to be the AMDV *VP2* gene (1944 nt; 2406–4349) with a BLAST search (BLAST, 2008). The insert showed the highest nucleotide (98%) and amino acid (97%) identities with AMDV-G (acc. no. M20036) and -SL3 (acc. no. X97629). The VP2 sequence of this Finnish AMDV strain, FIN05/C8, was deposited in GenBank (acc. no. GQ336866).

A protein band with the size of the AMDV VP2 protein (75 kDa) was identified with SDS-PAGE and Coomassie blue staining of the purified recombinant protein (Figure 1 in study II). Antigenicity was confirmed with a Western blot. Spontaneously-formed VLPs (23–25 nm in diameter) resembling parvovirus virions were observed under electron microscopy (Figure 3).

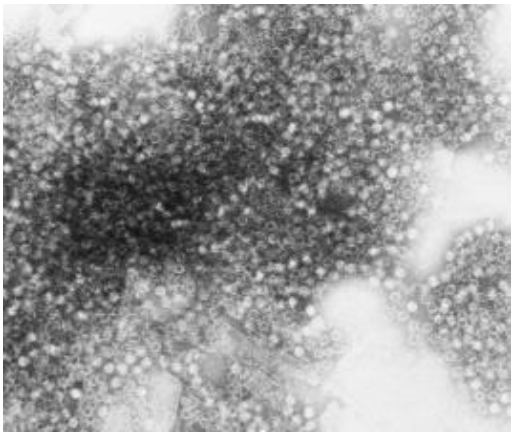

Figure 3. *Negative-stain electron micrograph of virus-like particles formed by the recombinant structural protein (VP2) of Aleutian mink disease virus.* Modified from study II, Figure 2, reprinted with the permission from ASM.

The purified antigen was tested with conventional CIEP and the newly-developed ELISA method. Serum samples from 209 mink were analyzed by CIEP using both the recombinant and the commercial antigen. The cross-classified results with the recombinant antigen showed a DSe and DSp of 100% when compared to the commercial antigen (Table 9). Serum samples from 316 mink were analyzed with ELISA using the recombinant antigen and CIEP using the commercial antigen. When compared to CIEP, the ELISA results had a DSe of 99% and DSp of 97%. For the ELISA OD values, see Figure 3 in study II.

Table 9. *Cross-classified results for CIEP performed with commercial antigen compared with the results of CIEP and ELISA performed with recombinant AMDV VP2 antigen*

		CIEP (commercial antigen)			Overall proportion agreement% (95% CI)	DSe% (95% CI)	DSp% (95% CI)
		Positive	**Negative**	**Total**			
ELISA (recombinant antigen)	**Positive**	104	6	110	98.0	99.0	97.2
	Negative	1	205	206	(94.5–98.4)	(95.6–99.9)	(94.2–98.8)
	Total	105	211	316			
CIEP (recombinant antigen)	**Positive**	100	0	100	100.0	100.0	100.0
	Negative	0	109	109	(98.8–100.0)	(97.5–100.0)	(97.7–100.0)
	Total	100	109	209			

Abbreviations: CIEP, counter-current immunoelectrophoresis; ELISA, enzyme-linked immunosorbent assay; AMDV, Aleutian mink disease virus; VP2, structural protein 2; DSe, diagnostic sensitivity; DSp, diagnostic specificity; CI, confidence interval. Modified from study II, reprinted with permission from ASM.

5.1.2. Validation of the automated ELISA (III)

The new ELISA method employing the recombinant VP2 antigen was automated and a new sampling method called 'blood comb' was developed. The new test was validated by comparing results with those of the CIEP test and according to OIE standards (OIE, 2008) including analytical, diagnostic and repeatability parameters.

Performance metrics of the ELISA and CIEP tests were comparable (Table 10). Only a few discordant results were found, resulting in similar DSe and DSp. Six discordant results (3 ELISA positive/CIEP negative, and 3 ELISA negative/CIEP positive) were found in the high prevalence population and three in the low prevalence population (ELISA positive/CIEP negative). For the ELISA OD values, see Figure 2 (histograms) and Figure 3 (box-and-whisker plot) in study III.

The median DSe of the ELISA test was 96.2% and DSp 98.4% according to a Bayesian analysis applying informative priors on CIEP test performance and prevalence (model 2) (Table 11 and study III, Table 3). The ELISA and CIEP had comparable accuracy in the two populations, as the probability that ELISA DSe and DSp were greater than the CIEP DSe and DSp were 58% and 55%, respectively. Results for models 1 and 3, modelling informative priors on a single parameter, were similar and verified the accuracy of the test. The dependence model was biologically and statistically more suitable for

Bayesian modelling as the conditional correlations (data not shown) between ELISA and CIEP results for models 1–3 were positive. Results of the other analyses included in the validation studies are given in Table 11.

Table 10. *Cross-classified results for CIEP and automated AMDV VP2 ELISA*

		CIEP		
		Positive	**Negative**	**Total**
ELISA	**Positive**	343	6	349
	Negative	3	409	412
	Total	346	415	761

Abbreviations: CIEP, counter-current immunoelectrophoresis; AMDV, Aleutian mink disease virus; VP2, structural protein 2; ELISA, enzyme-linked immunosorbent assay.

Table 11. *Results of the validation analyses of the automated AMDV VP2 ELISA.*

Analysis	**Result**	**95% CI**	**95% PI**
Analytical sensitivity	Detection limit: ELISA 1:10 000; CIEP 1:100 (for the titration curve, see Figure 4, study III)	NA	NA
Analytical specificity	No cross reactivity against mink enteritis virus, *Pseudomonas aeruginosa*, or *Clostridium botulinum.* Identified several Finnish, Swedish, and Estonian AMDV strains from different mustelid species (I, II, IV, Knuuttila, 2014 unpublished observations, Andersson and Wallgren, 2013).	NA	NA
Diagnostic sensitivity	96.2%	NA	91.5–99.0%
Diagnostic specificity	98.4%	NA	95.3–99.8%
Kappa	0.976	0.961–0.992	NA
Overall proportion agreement	98.8%	97.9–99.4%	NA
Proportion positive agreement	98.7%	97.7–99.8%	NA
Proportion negative agreement	98.9%	97.0–99.4%	NA
Within-run variability	Negative serum, CV 4% Low-positive serum, CV 8%	NA	NA
Between-run variability	Negative serum, CV 9% Low-positive serum, CV 26%	NA	NA
Between serial repeatability	Negative serum, CV 8% Low-positive serum, CV 6%	NA	NA
Positive predictive value	Farm 1, 99.9% Farm 2, 62.1%	NA	99.7–99.99% 6.5–69.0%
Negative predictive value	Farm 1, 61.2% Farm 2, 99.9%	NA	17.8–90.0% 99.6–99.99%
Prevalences	Farm 1, 94.3% Farm 2, 2.6%	NA	90.3–98.4% 0.3–5.4%

Abbreviations: AMDV, Aleutian mink disease virus; VP2, structural protein 2; ELISA, enzyme-linked immunosorbent assay; CIEP, counter-current immunoelectrophoresis; CI, confidence interval; PI, probability interval; NA; not applicable; CV, coefficient of variation.

5.2. Epidemiology and phylogenetics of AMDV in Finland (I, IV)

5.2.1. Serological and PCR results (I, IV)

Farmed mink (I)

To study the molecular epidemiology and construct phylogenetic trees of AMDV in Finnish farmed mink, organ and serum samples were collected from 17 animals from six farms. These samples were analyzed by CIEP for anti-AMDV antibodies and with NS1-PCR for AMDV DNA. From the sampled mink, 13 were AMDV-antibody positive and 14 AMDV-DNA positive. Three mink from farm A were negative. PCR results of each mink and organ are shown in Table 2 (study I). Some gross lesions typical for AD were detected in the positive mink, such as splenomegaly, mesenteric lympadenopathy, and discoloration and enlargement of the kidneys and liver.

Free-ranging mustelids (II)

Molecular and analytical tools were used to clarify the epidemiology of AMDV in free-ranging mustelids in Finland. Samples were collected from 308 animals and analyzed with the new ELISA test for antibodies and with the new NS1-qPCR for DNA. Anti-AMDV antibodies were found in 35/308 and AMDV DNA in 34/92 mustelid samples (see Table 3 in study IV). Nine discordant results were found, of which seven were from badgers (5 ELISA positive/PCR negative, 2 ELISA negative/PCR positive) and two from mink (ELISA negative/PCR positive).

Positive samples were detected in 3/8 species (see Figure 4 below and Table 1 in study IV). The prevalence was 54.4% in mink (31/57), 26.9% in badgers (7/26), and 7.1% in polecats (1/14). Pine martens (183), Eurasian otters (24), least weasels (2), wolverine (1), and stoat (1) had negative samples. Prevalence was higher in juveniles (19.2%) than in adults (10.2%), in females (13.6%) than in males (12.3%), and in years 2006–2009 (21.1%) than in 2010–2014 (7.0%) (Table 12). The differences were statistically significant ($P < 0.05$) in the species and year variables (Table 13 and Table 6 in study IV).

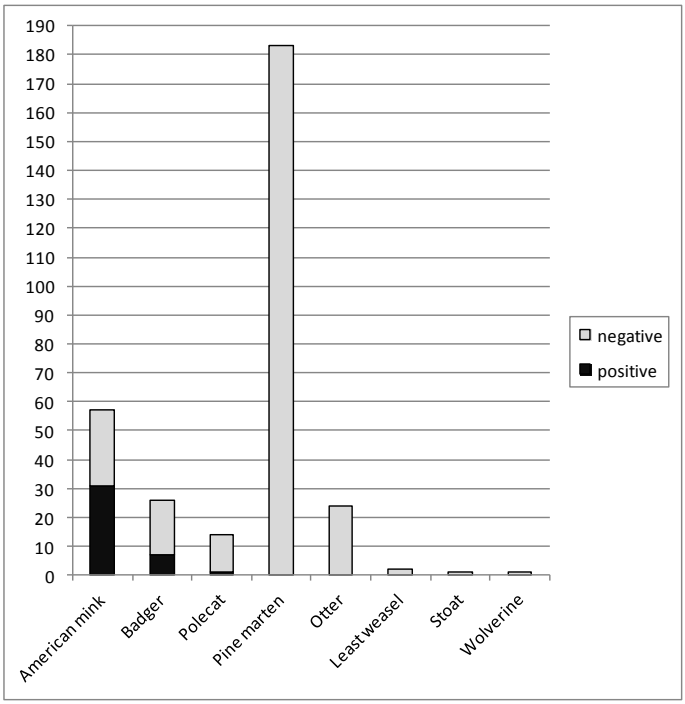

Figure 4. *Number of AMDV-infected and -non-infected (tested by ELISA and/or qPCR) individuals in Finnish free-ranging mustelid species during 2006–2014.* Abbreviations: AMDV, Aleutian mink disease virus; ELISA, enzyme-linked immunosorbent assay; qPCR, real-time quantitative polymerase chain reaction.

Table 12. *Number and percentage of AMDV-infected[a] mustelids per variable[b]*

Variable	Infected/sampled	%	95% CI
Species			
American mink	31/57	54.4	41.5–66.8
Badger	7/26	26.9	12.9–45.7
Other species	1[c]/225	0.4	0.0–2.1
Age			
Juvenile	15/78	19.2	11.7–29.0
Adult	11/108	10.2	5.5–16.9
Sex			
Male	24/195	12.3	8.3–17.5
Female	15/110	13.6	8.2–21.0
Year			
2006–2009	26/123	21.1	14.6–29.0
2010–2014	13/185	7.0	4.0–11.4
Municipalities, presence of mink farms			
No mink farms	33/283	11.7	8.3–15.8
Mink farms	6/25	24.0	10.7–42.9
Administrative regions, presence of mink farms			
No mink farms	14/64	21.9	13.1–33.1
Mink farms	25/244	10.2	6.9–14.5
Administrative regions, presence of mink farms			
No mink farms	14/64	21.9	13.1–33.1
≤15 mink farms	16/196	8.2	4.9–12.6
>15 mink farms	9/48	18.8	9.7–31.4

[a] ELISA and/or qPCR positive.
[b] All sampled regions (n = 17) were also included as a variable in the statistical analyses. Details are shown in Table 1 in study IV.
[c] Polecat.
Abbreviations: CI, confidence interval; AMDV, Aleutian mink disease virus; ELISA, enzyme-linked immunosorbent assay; qPCR, real-time quantitative polymerase chain reaction. Minor modifications from study IV. Reprinted with the permission from Society for General Microbiology (SGM).

Table 13. *Multivariable logistic regression results of factors associated with AMDV infection in Finnish free-ranging mustelids during 2006–2014*

Factor	B	S.E.	Wald's P-value	OR	95% CI
Species					
Mink	5.81	1.06	0.00	334.59	41.98–2666.91
Badger	4.30	1.11	0.00	73.61	8.40–644.78
Other species	0.00	NA	0.00	1.00	NA
Years					
2006–2009	1.66	0.50	0.00	5.28	1.97–14.13
2010–2014	0.00	NA	NA	1.00	NA
Constant	-6.36	1.07	0.00	0.00	NA

Abbreviations: AMDV, Aleutian mink disease virus; B, logistic regression coefficient; S.E., standard error of each coefficient; OR, odds ratio; CI, confidence interval; NA, not applicable.
Modified from study IV. Reprinted with the permission from SGM.

5.2.2. Geographic distribution of AMDV in the wild (IV)

Samples from free-ranging mustelids were obtained from 17 of the 19 administrative regions in Finland; no samples were available from Varsinais-Suomi or Åland (Figure 5). Positive samples were detected in 10 of the 17 sampled regions with a prevalence of 4.6–50.0% (see Table 1 in study IV and Figure 5). Prevalence in free-ranging mustelids was highest (>20%) in Päijät-Häme, Pohjois-Savo, South Ostrobothnia, South Karelia, and Uusimaa. The AMDV prevalence was lower in those regions that had mink farms (8.2–18.8%), in contrast to those regions that had no farms (21.9%) (Table 12). At the municipal scale, prevalence was higher in municipalities with mink farms (24.0%) than in those without (11.7%). The differences in the prevalence were statistically significant ($P < 0.05$) in two comparisons; regions with mink farms versus no mink farms, and regions with less than 15 farms versus no mink farms (see Table 6 in study IV).

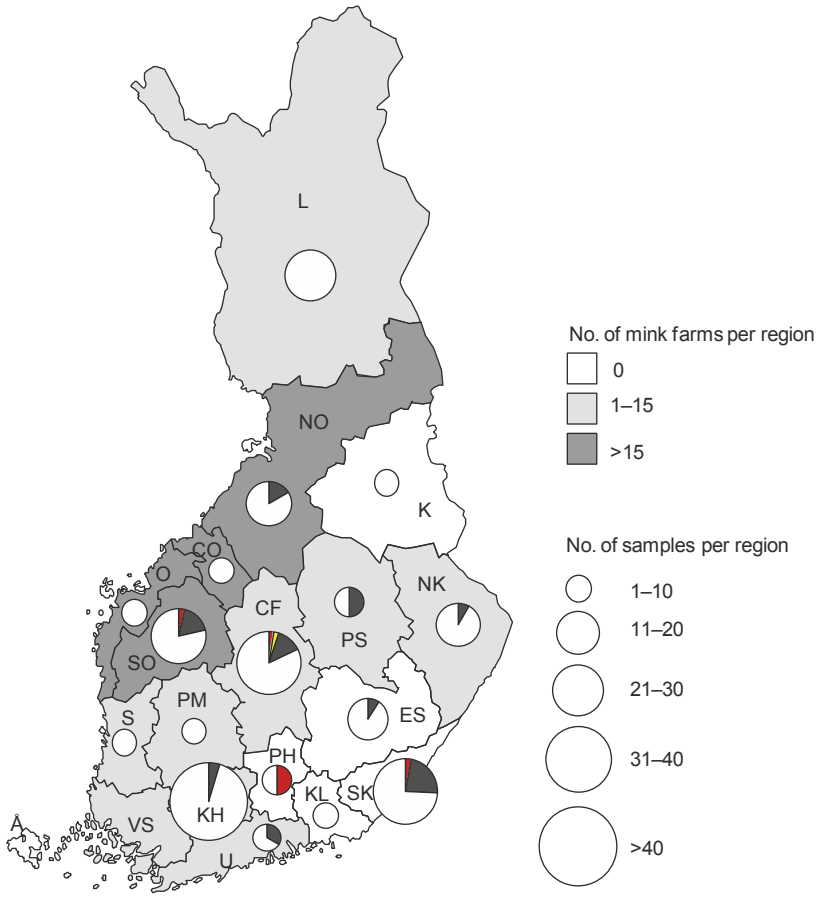

Figure 5. *Prevalence of Aleutian mink disease virus in free-ranging mustelids and the number of mink farms per administrative region in Finland during 2006–2014.* The size of the pie charts represents the total number of samples; black, percentage of positive American mink samples; yellow, percentage of positive polecat samples; red, percentage of positive badger samples; white, percentage of negative samples. Shading of the region represents the number of mink farms per region during the twenty-first century. Abbreviations: U, Uusimaa; VS, Varsinais-Suomi; S, Satakunta; KH, Kanta-Häme; PM, Pirkanmaa; PH, Päijät-Häme; KL, Kymenlaakso; SK, South Karelia; ES, Etelä-Savo; PS, Pohjois-Savo; NK, North Karelia; CF, Central Finland; SO, South Ostrobothnia; O, Ostrobothnia; CO, Central Ostrobothnia; NO, North Ostrobothnia; K, Kainuu; L, Lapland; Å, Åland.© National Land Survey of Finland (NLS) 2014 (Contains data from the NLS municipal division database 6/2014). Minor modifications from study IV. Reprinted with the permission from SGM.

5.2.3. Factors associated with AMDV infection in free-ranging mustelids (IV)

In the multivariable logistic regression, species and year of sample collection were identified as factors being associated with infection with an odds ratio (OD) of 335 for mink and 74 for badger when compared to other species and 5 for the 2006–2009 sampling period when compared to 2010–2014 (Table 13). Age, sex, location (region), or the presence of mink farms in the area (municipality, region) were not recognized as risk factors.

The final model was statistically significant and fitted the data well (Omnibus test of model coefficients, $\chi^2 = 124.68$, $P < 0.0005$; Hosmer and Lemeshow test, $\chi^2 = 0.341$, $P = 0.843$), accounting for 62.5% (Nagelkerke's R^2) of variance in infection. The model classified 88.3% of cases correctly with a cut-off of 0.3. Sensitivity of the model to predict infection was 94.9%, specificity 87.4%, positive predictive value (PPV) 52.1%, and negative predictive value (NPV) 99.2%. The area under the curve (AUC) for the model was 0.947 (95% CI 0.921–0.974, $P < 0.0005$). One outlier (studentized residual >2.5), the only infected polecat, was found and it was kept in the model. No confounders or one-level interactions were found.

5.2.4. Sequence analysis (I, IV)

In study I, 14 new AMDV-NS1 sequences (343–377 nt in length) were obtained from Finnish farmed mink and deposited in GenBank (see Appendix I for accession numbers). Forty additional AMDV sequences, containing four previously-isolated Finnish strains (Olofsson et al., 1999), were obtained from GenBank and included in the analyses. Mean nucleotide sequence identities of all AMDV strains were 89% and amino acid identities 83% (see Table 3 in study I and Table 14). When the sequences within each phylogenetic cluster (more data in the next Chapter) were compared, the variability was lower, being 93–97% for nucleotide and 89–94% for amino acid data. The new Finnish strains had 86–100% nucleotide and 78–100% amino acid identity to each other. The most divergent sequences of these new Finnish sequences (FIN05/D11–12 vs. FIN05/B4–6) had 86% nucleotide and 78% amino acid identity. Two sets of identical sequences were found (FIN05/B5 and FIN05/B6; FIN05/E14, FIN05/E15, and FIN05/F17). The mean d_S/d_N ratio was 2.71 (1.01–5.02), indicating a mild purifying selection (see Table 3 in study I).

In study IV, 20 new NS1 sequences (401–439 nt in length) were obtained from Finnish free-ranging mustelids, including 17 feral mink, 2 badgers, and 1 polecat and representing 9 administrative regions. Sequences were deposited in GenBank (see Appendix I for accession numbers). An additional 22 AMDV strains, representing Estonian free-ranging mustelids (4 badgers, 3 pine martens, 2 feral mink, 1 polecat) and 12 Finnish farmed mink were sequenced. Also, five global farmed mink strains were obtained from GenBank and included in the analyses. Nucleotide identities of all strains (both from farmed mink and free-ranging mustelids) ranged between 81% and 100% (Table 14). Identical sequences were found in Finnish farmed and feral mink and a badger (FIN14/L24 and FIN13/K22; Meme360_CF08 and Nevi358_CF08; Nevi456_U13 and Nevi458_U13). The most divergent sequences, with nucleotide identity of 81–86% to any other AMDV sequence, were found in four Estonian badgers. Initially, it seemed that sequences from free-ranging mustelids were more variable than those from farmed mink. However, the differences became trivial when the divergent sequences from badgers were excluded from the analysis.

Table 14. *Mean sequence identity[a] of partial AMDV NS1 gene in farmed mink and free-ranging mustelids*

Sequences from	N	Nucleotide identities	Amino acid identities	Reference
All farmed mink	54	89% (81–100%)	83% (70–100%)	I
All farmed mink and free-ranging mustelids	47	91% (81–100%)		IV
All farmed mink	17	93% (88–100%)		IV
Finnish farmed mink	12	93% (88–100%)		IV
All free-ranging mustelids	30	91% (82–100%)		IV
Free-ranging mustelids, excluding divergent sequences from Estonian badgers[a]	26	93% (88–100%)		IV
Finnish free-ranging mustelids	20	94% (88–100%)		IV
Estonian badgers[b]	4	97% (96–97%)		IV

[a] Calculated by pairwise comparisons.
[b] Meme125_EE07, 132_EE08, 133_EE08, and 134_EE08.
Abbreviations: AMDV, Aleutian mink disease virus; NS1, non-structural protein 1. Modified from studies I and IV. Reprinted with the permission from Elsevier and SGM.

5.2.5. Molecular epidemiology and phylogenetics of AMDV (I, IV)

For AMDV strains in farmed mink the MP (see Figure 2 in study I) and ML (data not shown) methods recovered a similar tree topology. The AMDV strains formed three phylogenetic clusters or groups and, as no official genotyping system currently exists (ICTV, 2014), they were designated Groups I, II, and III. Finnish AMDV strains were present within each of them. The newly-sequenced Finnish AMDV strains resolved in Groups I and II. Group I was formed of strains from farm D, thus diverging from other Finnish strains found in this study, Swedish AMDV strains, and the highly pathogenic United strain from the USA. Strains from farms B, C, E and F shared a common ancestor with AMDV-G (USA), -SL3 (Germany), highly pathogenic -Utah 1 (USA), and two previously described Finnish strains (Olofsson et al., 1999) forming Group III. Group II only contained Nordic (Finnish, Danish, and Swedish) AMDV strains. The strains in Group III seemed to be more conserved with shorter branches than strains in the other clusters. Within the studied region, no recombination was detected between clusters (data not shown) and a clock-like substitution rate was rejected.

In study IV, the ML (Figure 6) and Bayesian (data not shown) methods recovered similar tree topology for AMDV strains obtained from several mustelid species. The AMDV strains formed five phylogenetic clusters, with strains from free-ranging mustelids in each. Finnish AMDV strains could be found in 4/5 (I–IV), strains from farmed mink in 4/5 (I–IV), and strains from free-ranging mustelids in 5/5 (I–V) groups. Group I of both studies (I and IV) and Group III of study I and Group II of study IV are equivalent and share the same strains. Strains from Finnish farmed mink were mainly found in Groups I and II (one strain in Group IV) and strains from Finnish feral mink in Groups I and III. Finnish strains from other mustelid species were resolved in three groups (I, III, and IV). In Groups I and IV, strains from free-ranging mustelids from Finland and Estonia shared a common ancestor with strains from Finnish farmed mink. AMDV strains from Estonian feral mink and Finnish farmed mink shared a common ancestor with global strains from

farmed mink in Group II. Group III was formed by strains from Finnish and Estonian free-ranging mustelids and from strains from Chinese farmed mink. Most interestingly, Group V was formed solely by divergent Estonian badger AMDV strains (Figure 6). Although the virus was variable, it seemed to be evolving quite slowly, as strains isolated from Finnish farms up to nine years apart (Groups I and II), had not acquired new substitutions indicated by short terminal branches. Except for the divergent badger sequences and the tendency of strains from Finnish farmed and feral mink to diverge into separate clusters, no major factor linked to host species, location, isolation year, or pathogenicity could be detected in either study (I, IV). The phylogenetic analyses (I, IV) infer at least three introductions of AMDV into Finnish mink farms, as the Finnish strains currently circulating in farmed mink were resolved in three phylogenetic groups.

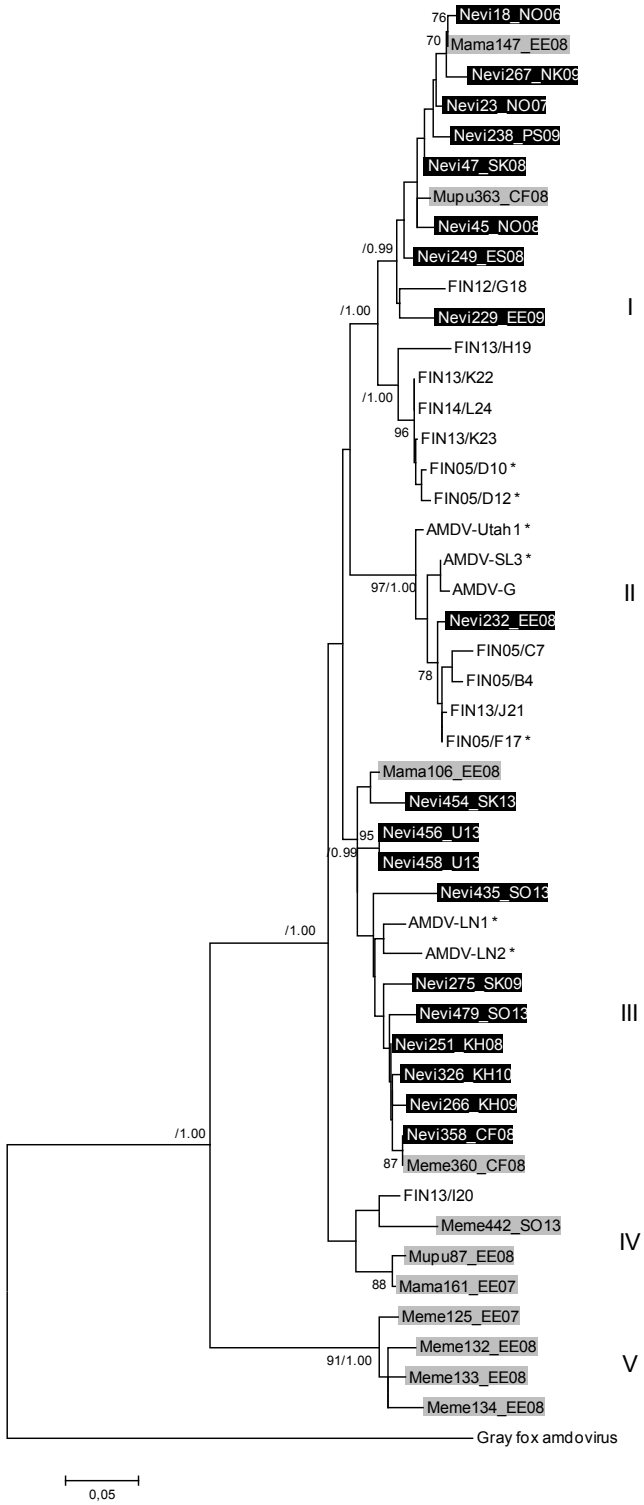
Nevi18_NO06
Mama147_EE08
Nevi267_NK09
Nevi23_NO07
Nevi238_PS09
Nevi47_SK08
Mupu363_CF08
Nevi45_NO08
Nevi249_ES08
FIN12/G18
Nevi229_EE09
FIN13/H19
FIN13/K22
FIN14/L24
FIN13/K23
FIN05/D10*
FIN05/D12*
AMDV-Utah1*
AMDV-SL3*
AMDV-G
Nevi232_EE08
FIN05/C7
FIN05/B4
FIN13/J21
FIN05/F17*
Mama106_EE08
Nevi454_SK13
Nevi456_U13
Nevi458_U13
Nevi435_SO13
AMDV-LN1*
AMDV-LN2*
Nevi275_SK09
Nevi479_SO13
Nevi251_KH08
Nevi326_KH10
Nevi266_KH09
Nevi358_CF08
Meme360_CF08
FIN13/I20
Meme442_SO13
Mupu87_EE08
Mama161_EE07
Meme125_EE07
Meme132_EE08
Meme133_EE08
Meme134_EE08
Gray fox amdovirus
I
II
III
IV
V
0,05

Figure 6. *A phylogenetic tree of Aleutian mink disease virus (AMDV) sequences from 30 free-ranging mustelids and 17 farmed mink based on an alignment of 373 nt fragment, including the end of the NS1 gene and part of the non-coding region, corresponding to nt 1854–2230 of the AMDV-G sequence (JN040434).* The tree was reconstructed with a maximum-likelihood method and the numbers at nodes show bootstrap values (1000 pseudoreplicates, ≤70% are not shown), and Bayesian posterior probabilities (right-hand values). Roman numerals indicate phylogenetic groups recognized in this study. The scale for genetic distance is provided. Known pathogenic strains are marked with an asterisk, feral mink strains with a black background, indigenous wild mustelid strains with a gray background, and farmed mink strains with a white background. Abbreviations: NS1, non-structural protein 1; nt, nucleotide. For strain abbreviations see Appendix I. Minor modifications from study IV. Reprinted with the permission from SGM.

6. Discussion

6.1. Diagnostics (II, III)

6.1.1. New recombinant VP2 antigen can be used both in CIEP and in ELISA (II)

In this study (II), a recombinant AMDV VP2 antigen based on a Finnish field strain was engineered and used to develop an indirect IgG ELISA test. Results showed that the recombinant VP2 proteins were antigenic, formed VLPs, and could be used as an antigen both in a conventional CIEP test (DSe and DSp, 100%) as well as in the new ELISA test (DSe, 99%; DSp, 97%).

Aleutian mink disease virus recombinant VP2 or VP1/VP2 proteins have been made previously (Clemens et al., 1992; Christensen et al., 1993; Wu et al., 1994; Costello et al., 1999; Zeng et al., 2007), and have been shown to be antigenic and form VLPs (Clemens et al., 1992; Christensen et al., 1993; Wu et al., 1994). In spite of these early developments, diagnostic applications have been scarce (Clemens et al., 1992; Aasted et al., 1998; Zeng et al., 2007) and published data on their performance are limited. Clemens et al. (1992) showed that the recombinant VP1/VP2 antigen gave higher DSe and higher titers in CIEP than the cell-culture derived AMDV-G antigen (n = 10). Zeng et al. (2007) reported a DSe of 100% and DSp of 90.5% for CIEP performed with the recombinant VP2 antigen when compared to the cell-culture derived antigen (n = 54). We also attempted traditional propagation of the virus (Finnish strain FIN05/B4) in a cell culture, but replication failed after a few passages (Knuuttila, 2005 unpublished observations). Instead, expression of the recombinant protein in the baculovirus allowed for fast and easy production of large amounts of conformationally ideal antigen (VLPs).

Several studies have described ELISA tests for detecting anti-AMDV antibodies in mink (Wright and Wilkie, 1982; Miroshnichenko et al., 1992; Aasted et al., 1998; Costello et al., 1999; Castelruiz et al., 2005), but until recently (see the next Chapter) these were mainly used for research instead of mass screening purposes. Data on the accuracy of the ELISA tests have also been scarce. In one study, results of ELISA and CIEP tests using an organ-derived whole virus antigen were compared and the authors concluded that the DSe of ELISA is rather low (Wright and Wilkie, 1982). In contrast, a later study found ELISA to be more sensitive than CIEP (Miroshnichenko et al., 1992). Although the CIEP and ELISA tests performed similarly in our study (II), a few discordant results were observed. One sample was determined to be negative by ELISA but positive by CIEP, whereas six samples were pronounced positive by ELISA but negative by CIEP. Several studies suggest that the ASe and/or DSe of CIEP is lower than that of other methods (Crawford et al., 1977; Aasted and Cohn, 1982; Alexandersen and Hau, 1985; Alexandersen et al., 1985a; Aasted et al., 1986; Miroshnichenko et al., 1992; Farid et al., 2015). Thus, it is possible that at least some of the ELISA-positive but CIEP-negative samples were actually true positives, meaning that the ELISA would be more accurate than these results

suggested. This is further supported by the rather high ODs (0.6–1.2) of most (4/6) of the discordant samples (see study II, Figure 3). However, false positive results can be caused by a variety of reasons, for example cross-reactions, non-specific binding, differences in the antibody detection level and in the capability of antibodies to recognize different AMDV strains. Furthermore, differences in the structure of the antigen (CIEP, whole virus; ELISA, recombinant VP2) and in the propagation and purification methods may account for some discrepancies in the results.

Due to the implementation of the eradication program in Finland in 2005, the number of tested samples rose from 330 000 in 2005 to nearly 800 000 in 2014 (M. Eerola [Fin Furlab], personal communication 21.5.2015). The anticipated rise in sample numbers created a need for a highly accurate, economical, and high-throughput assay, as CIEP test requires a large amount of labor and is not well-suited to automation. During development of the ELISA assay system, technical challenges emerged and dispensing several assay reagents had to be automated and a new system of collecting and transferring blood/serum samples to an ELISA plate had to be created. Results of this study (II) showed, however, that the traditional antigen can be replaced by the newly-developed recombinant antigen and encourages further development of the ELISA assay for mass screening of AMDV infection in mink.

6.1.2. New automated ELISA test for the serodiagnosis of AMDV in mink (III)

The new ELISA test based on the VP2 recombinant antigen and utilizing the new 'blood comb' sampling method was automated and validated (study III). The test was compared to conventional CIEP with cell culture antigen (Danad) and glass capillary sampling. The ELISA test was found to be highly sensitive (DSe 96.2%), specific (DSp 98.4%) and suitable for the large-scale serodiagnosis of AMDV infection in mink.

Diagnostic tests used for the eradication and control of a disease should be reviewed and updated at regular intervals. If one test is used for an extended period of time, with decreasing prevalence the probability that false-positive animals will be culled increases. As the CIEP test has been in widespread use for several decades, an updated or new test for the serodiagnosis of AMDV was deemed necessary. Serological assays offer an appropriate technique for diagnosing AMDV infection in mink, as presumably the level of anti-AMDV antibodies is high in all infected adults even if they do not develop chronic disease symptoms (Bloom et al., 1994). In eradication programs in general, a screening test with a high DSe and ASe is needed to identify positive animals in early stages of infection so control measures can be taken to limit the disease spreading. However, if no confirmation test is used, the test should also have high DSp (and thus high PPV at low prevalence) to avoid false-positive results and excessive culling and costs to farmers (in the case of AMDV: loss of valuable breeding animals and group A status, and effects on mink trade). Our results show that the new automated ELISA test can be utilized for the control and eradication of AMDV, calculating seroprevalence, and confirming the infection status of farms and individual mink. The test was first implemented at Fin Furlab in 2008, and since that time the testing capacity has increased and the sample processing time has decreased. Furthermore, although capital investment was needed for new

equipment, testing costs are now lower due to decrease in labor cost. Moreover, the new ELISA system also allows automated plate reading and data management. In contrast, CIEP results must be read by a human operator and are thus subject to error before being recorded manually and stored in a paper format.

The results of our study (III) on the DSe and DSp of the automated ELISA test are comparable to the findings of Andersson and Wallgren (2013) and our previous study (II). Farid and Segervall (2014) reported slightly lower DSe (91% and 94%) for our ELISA compared to CIEP, but their results fell within the 95% PI reported by us in study III. In our study (III), results of the Bayesian analysis showed that the DSe and DSp of ELISA and CIEP were not significantly different irrespective of the model used, and were comparable for both tests. Nine discordant results were found between ELISA and CIEP (Table 10). As in study II, most (6) of these were ELISA-positive but CIEP-negative. The possible causes of the discordance have been detailed in the previous chapter. This test, as far as is known, is the first automated ELISA method (in use since 2008) for the serodiagnosis of AMDV infection in mink. Recently, few other commercial ELISA tests have been developed. A Danish automated ELISA test, based on cell-culture derived AMDV-G antigen (ELISA Danad antigen, Kopenhagen Fur, Glostrup, Denmark) and dried blood spot cards, was shown to have a similar performance to CIEP with DSe of 88.6% and DSp of 99.8% (Dam-Tuxen et al., 2014). In addition to this, an ELISA kit based on the cell-culture derived AMDV-G antigen (Scintilla Development Company LLC, Bath, PA, USA) is available. Andersson and Wallgren (2013) compared the results of this kit and the ELISA test developed by us to CIEP and concluded that our recombinant VP2-ELISA had higher DSe than the ELISA kit. They reported a DSe of 99.7% and DSp of 98.3% for our ELISA when compared to CIEP. Additionally, an ELISA test is available for ferrets at the University of Georgia, USA (http://www.vet.uga.edu/idl/tests).

The results suggested high ASe and ASp for ELISA. As in several previous studies of other test methods (Crawford et al., 1977; Aasted and Cohn, 1982; Alexandersen and Hau, 1985; Alexandersen et al., 1985a; Aasted et al., 1986; Farid et al., 2015), our findings also infer that the ASe for CIEP is lower than that for ELISA. In general, the analytical sensitivity of ELISA test (approximate sensitivity <1 µg/dl) is higher than that of counterimmunoelectrophoresis (<0.1 mg/dl) (reviewed by Thrusfield, 2007b). However, for more reliable results of the ELISA ASe, additional samples, replicates, and dilutions should have been made and examined. Although the strains of AMDV are highly variable (study I and IV, Gottschalck et al., 1994; Olofsson et al., 1999) , this ELISA test was able to identify positive samples from mink infected with strains from different groups and countries as well as several species (studies I, II, and IV, Andersson and Wallgren, 2013, Knuuttila, 2014 unpublished observations). On the other hand, positive reactions caused by antibodies directed to other mink pathogens were not seen.

In general, repeatability of the ELISA test was high with a CV less than 10%. One exception was the low-positive serum with a between-run CV of 26%. The higher variability of this sample was anticipated, as sera with values between the cut-off and high-positive value tend to vary more than the negative and high-positive samples. As this test is used qualitatively and not quantitatively, and none of the low-positive values were below the cut-off point, this was not regarded as a major problem. When further

developing this test platform, this issue can be taken into account by normalizing the results, for example, by expressing them as S/P-ratio (as in study IV) and by implementing an internal quality control system such as Levey-Jennings control charts or Westgard multirule system (Westgard, 2009).

As expected, the PPV was high in the high prevalence population and the NPV for the low prevalence population. However, despite the rather high DSp, the PPV was only 62.1% for the low prevalence population. This issue can be resolved by using confirmatory tests (i.e., serial testing) with a high DSp for those animals that test positive in the ELISA test, especially in AMDV-free and low prevalence farms. Primers and a qPCR protocol, described in study IV, are currently used for this purpose to confirm AMDV DNA positivity. A sample from the spleen is required for reliable diagnostic results (Jensen et al., 2011), as excretion in feces, saliva, and blood often appears to be intermittent and transient (Jensen et al., 2014; Farid et al., 2015). Anyhow, the duration of the excretion needs further research as the results might reflect the difference in ASe of the different PCR protocols. Optimally, the confirmatory test would not require the animal to be sacrificed.

In Finland, the ELISA test is routinely used at Fin Furlab and the system is also used in the Netherlands. The recombinant VP2 antigen is commercially available and used for diagnostic purposes in Sweden and the Netherlands. The test may also be used to study the epidemiology of AMDV in mink and free-ranging mustelids. Efforts to produce a commercially-available ELISA kit based on this system are also under way (Poikulainen, 2014). As the ELISA test also provides quantitative data, it might be possible to develop a classification scheme based on the results (Farid and Segervall, 2014). Animals with high OD values fall into different classes than animals with low OD values. This might help the farmer to cull mink that are more seriously affected. Further knowledge, however, is needed whether feasible antibody classes can be implemented and whether these classes actually interrelate with the severity of the disease.

6.1.3. Filter-paper blood comb – a new sampling method for ELISA (III)

A new sampling method, filter paper blood comb (see study III, Figure 5), was developed to replace the glass capillary tubes used in CIEP. Additionally, a specific device (see study III, Figure 6) was engineered to transfer samples on the blood combs to an ELISA plate. The device ensures rapid and precise handling and simultaneous elution of samples preventing variation in results caused by differences in the elution and incubation times. Guthrie and Susi (1963) first introduced the filter paper blood sampling for infants in the 1960s. Since then, it has been applied in human and veterinary medicine (Mei et al., 2001; reviewed by Curry et al., 2011). However, in many of the ELISA tests employing filter paper sampling, an additional elution and dilution step of the blood samples is required. In our method, the comb can be directly introduced to the ELISA plate for the elution of antibodies, thus reducing the processing time. Blood comb sampling offers several advantages: the required blood sample is small and collection is simple and rapid; samples do not get mixed, lost, or damaged; they are easy to code, ship, and store, and the centrifugation step required by capillary tubes systems is not needed. Blood combs also

make it possible to pool several samples into a single well. This testing scheme is particularly suitable for the monitoring of AMDV-free farms, reducing costs and allowing for rapid and more frequent testing.

6.2. Epidemiology and phylogenetics of AMDV in Finland (I, IV)

6.2.1. Several introductions into Finnish farms (I, IV)

Based on the tree topology, the AMDV strains formed five genetic groups, of which strains from farmed mink were present in four. However, the fifth group, due to its divergence, possibly represents a new and currently undescribed species (Figure 6). Previous studies have reported three to six phylogenetic groups for AMDV (Olofsson et al., 1999; Jensen et al., 2012; Sang et al., 2012; Nituch et al., 2012; Leimann et al., 2015). It appears that AMDV has been introduced into Finnish farms on several occasions. At least three introductions have occurred, as strains from Finnish farmed mink could be found in three phylogenetic clusters. A fourth introduction is also possible, as closely related strains were found in Finnish free-ranging mustelids and Chinese farmed mink (Group III, study IV), suggesting a common ancestor in Finnish farmed mink. But this lineage might have disappeared from farms (e.g., due to eradication efforts) or was not found due to a limited number of samples.

The variability of Finnish strains suggests that the origins of the Finnish virus strains are in several sources. Unfortunately, the exact origin of the Finnish strains (both at farms and in the wild) remains to be clarified due to a lack of data on the animal trade and the limited number of available sequences from exporting countries and wild mustelids elsewhere. Some inferences can, however, be made. As Finnish strains shared ancestors with strains from farmed mink from several different countries (the USA, Denmark, Sweden, China, Estonia, and Germany), it may be hypothesized that the virus has been introduced into Finnish farms through the global mink trade. It is known that the first mink were imported from the USA during the 1930s and subsequently from Denmark and Sweden in the 1940s. Thus, the Finnish strains in Groups III (study I) and II (study IV), containing two strains from the USA, may be of North American origin. The ancestors of Groups I (study I and IV) and II (study I) might originate from other Nordic countries as these included several sequences from Sweden and one from Denmark. Fewer data are available from Groups III and IV (study IV). Subsequently, the virus may have spread into the wild either from infected escaped/released mink from Finnish farms and at the Russian border or from the premises of infected farms accessed by wild animals. Alternatively, it might also have been present in wild mustelids before mink farming began. Wild mustelids may also carry their own divergent strains, potentially new amdoparvoviruses, as observed in Estonian badgers. In summary, the majority of the Finnish AMDV strains are closely related to other European and North American AMDV strains and strains of various genotypes are present in Finland. The situation is different in Denmark where the virus population seems to be more homogenous, which is probably due to long lasting

eradication efforts that have been in place since 1976 (Christensen et al., 2011). Also, the origin of the common ancestor of all AMDV strains needs further study. Due to historic reasons (e.g., North American origin of mink and the first appearance of the disease) it may be hypothesized that AMDV has been transmitted from North America to the other mink-producing countries, although this may be too simplistic (Farid, 2013). Instead of mink, origin of AMDV in another mustelid species is also possible.

In our studies, a partial *NS1* gene was analyzed and more sequence data (both larger number and longer sequences) are needed to further elucidate the evolutionary history and relationships of this virus on a global scale. In particular, it would be interesting to study the presence of AMDV or related amdoparvoviruses in farmed mink, ferrets, and wild mustelid species in other countries and especially those with no history of mink farming. In study I, we used the same partial *NS1* gene as Olofsson et al. (1999) to include as many reference sequences as possible. At the time, the amount of AMDV sequence data in GenBank was very limited. The initial plan in study IV was to use both partial NS1 and partial VP2 (Oie et al., (1996), as at the time many reference sequences were available for mink, less so for other mustelids. However, we soon noticed that these protocols failed to identify all AMDV strains and a new qPCR protocol with new primers was applied (see Chapter 6.2.5.). Unfortunately, a disadvantage met with in doing this was the restricted number of global reference sequences. As our focus also included the analytical epidemiology, we sought to sequence as many infected individuals as possible.

Phylogenetic trees recovered in our studies included several new field strains with nucleotide data and discrete phylogenetic methods. We aimed to use the most sophisticated phylogenetic methods available and to take all codon positions into account to analyze the evolutionary differences more sensitively. The resolution of previously-described strains (United, K, SL3, Utah 1, G, and Finnish and Swedish strains in Olofsson et al. (1999)) was similar to what has been presented earlier (Gottschalck et al., 1994; Schuierer et al., 1997; Olofsson et al., 1999). Also, the grouping of strains from farms B, C, D, and F, shared by both studies, was comparable.

Although the focus of these studies was to infer the molecular epidemiology and evolutionary relationships of Finnish AMDV strains, results provided additional insights. Building on Oloffson et al. (1999), study I represents one of the first studies of the molecular epidemiology of AMDV and contained many more sequences than previous works. Since then, the extent of phylogenetic research of AMDV has developed hand-in-hand with advances in molecular techniques and phylogenetic and statistical methods of analysis. Thus, in the last five years, a growing number of sequences exists for feral and wild mink and the number of studies elucidating the molecular epidemiology of the virus has increased (Christensen et al., 2011; Jensen et al., 2012; Nituch et al., 2012; Sang et al., 2012; Leimann et al., 2015; Nituch et al., 2015). However, study IV was the first description of the molecular epidemiology of this virus in mustelids other than mink and ferret. The new sequence data obtained and detection methods developed for these studies enable further research on the epidemiology and evolutionary relationships of AMDV and related viruses as well as outbreak investigations at farms. Additionally, the qPCR can be used as a confirmatory test.

6.2.2. Strains show high variability (I, IV)

Based on the partial NS1 sequence, the Finnish AMDV strains from farmed mink showed divergence of up to 14% at the nucleotide and 22% at the amino acid level, whereas the strains from free-ranging mustelids showed divergence of up to 12% at the nucleotide level. Identical sequences were identified both in the farmed mink within the same and from different farms and between feral mink and a badger. The overall variability of all AMDV strains was up to 19% at the nucleotide and 30% at the amino acid level, and similar to what has been reported previously for the NS1 region (Olofsson et al., 1999; Nituch et al., 2012). The most divergent mink strains were the Swedish strains D1S and N2S reported by Olofsson et al. (1999). The strains in these studies were quite similar within one farm, but strains from one farm can fall into different phylogenetic groups (Olofsson et al., 1999) and even one animal may be simultaneously infected with several types of strains (Gottschalck et al., 1991).

Although the virus was variable, it seemed to be evolving quite slowly, as strains that were isolated for several decades (Utah 1, 1960s; G, 1970s; K and SL3, 1980s) have failed to acquire many new mutations during that time. Similarly, strains from Finnish farms isolated for up to nine years remain unchanged (study IV, Groups I and II). Similar results concerning strain stability have been reported by Christensen et al. (2011), and Gottschalck et al. (1994) concluded that AMDV is an old virus based on retained nucleotide sequence substitution rate of canine parvovirus. However, study I rejected a molecular clock which provides no evidence for a general and linear evolutionary rate in AMDV. This makes it difficult to infer the age of the virus and place its diversification within an absolute timescale. Given that DNA viruses such as AMDV replicate in the nucleus and are subject to host-cell proofreading, they probably have mutation rates similar to that of host-cell DNA (reviewed by MacLachlan and Dubovi, 2011). Based on the NS1 sequence, AMDV appeared to be under mild purifying selection (mean d_S/d_N = 2.71). Mild purifying or neutral selection for AMDV NS1 has also been reported by others (Gottschalck et al., 1994; Lukashov and Goudsmit, 2001; Christensen et al., 2011), and the d_S/d_N ratio reported here is within the range known for other parvoviruses, i.e., 1 to 22.5 (Lukashov and Goudsmit, 2001).

Although it is known to occur in parvoviruses (Lukashov and Goudsmit, 2001; Shackelton et al., 2007; Wang et al., 2012; da Costa et al., 2013), recombination was not detected in our study (I), possibly due to the relatively short length of sequence studied. Although data are lacking, AMDV offers good opportunities for recombination (chronic infection, potential superinfections) and some authors suggest that it is likely (Shackelton et al., 2007; Christensen et al., 2011).

6.2.3. Potential novel amdoparvovirus in Estonian badgers (IV)

A highly divergent strain was discovered in Estonian badgers showing only 81% to 86% nt similarity to any other AMDV sequence based on the partial *NS1* gene and formed a sister group to other AMDV strains (Figure 6). It is possible that these Estonian strains represent a novel amdoparvovirus species, a ‘badger amdoparvovirus’. Further support for

this hypothesis was given by a median joining network analysis, which indicated that these four badger sequences were at least 2.5-times (gray fox amdovirus 3.8-times) more distant from their closest neighbor compared to 'regular' AMDV genotypes (Saarma, 2014 unpublished observations). However, more sequence data are needed to establish that the badger strain is sufficiently distinct to warrant recognition as a new species. Work involving next-generation sequencing is underway. In addition to the badgers in Estonia, it is possible that new amdoparvoviruses may also be found in indigenous mustelids of Finland.

For species other than mink and ferret, it is often the case that viruses (AMDV or related) causing the infection and antibody response are poorly documented and information on their evolutionary relationships is limited. Ferrets seem to carry viruses that are divergent from mink strains both biologically (Porter et al., 1982) and genetically (<90% nt identity to mink AMDVs, partial VP2) (Saifuddin and Fox, 1996; Murakami et al., 2001). Phylogenetic analysis resolved strains from ferrets in a distinct cluster (Murakami et al., 2001). In the wild, in addition to mink, similar AMDV strains (cross-species transmission) are carried by polecats, pine martens, and badgers (study IV), as well as by raccoons (Oie et al., 1996). Additionally, divergent strains seem to exist in other mustelids and carnivores. Nituch et al. (2015) showed that Canadian striped skunks carry strains that form a separate lineage from feral and wild mink strains having 82% to 91% nt identities (partial NS1) to mink strains. European mink and Eurasian otters may also carry AMDV strains that diverge from mink strains (Mañas et al., 2001). Notably, new amdoparvoviruses have recently been found in raccoon dogs and foxes (Li et al., 2011; Shao et al., 2014; Bodewes et al., 2014a). Amdoparvoviruses in raccoon dogs and arctic foxes show 82% (Shao et al., 2014) and gray foxes 74% nt identities (complete NS1) to mink strains (Li et al., 2011). No official species or strain-demarcation criteria currently exist for amdoparvoviruses (Tijssen et al., 2011). Generally, different species belonging to genera in the *Parvovirinae* subfamily are <95% related within the NS1 DNA sequence, are antigenically distinct, and are found predominantly in a single natural host species (Tijssen et al., 2011). A recent proposal to update the taxonomy of Parvoviridae requires the same species to show over 85% identity in the aa sequence of NS1 (Cotmore et al., 2014). Aleutian mink disease virus strains share over 87% nt and 82% aa identities within the *NS1* gene (Li et al., 2011), thus the proposed criterion seems more suitable for AMDV.

6.2.4. No clustering according to pathogenicity, year, or location (I, IV)

Similar to the findings of previous studies (Schuierer et al., 1997; Olofsson et al., 1999; Nituch et al., 2012; Leimann et al., 2015), we found no clusters that associated with pathogenicity, year of isolation, or geographical origin. Known pathogenic strains were identified in almost all clusters and they appear to have independently evolved from less pathogenic clusters. Thus, a molecular basis to explain the AMDV outbreaks that occurred in 2004 and 2005 at farms D, E, and F could not be found (I). Furthermore, no inferences can be made on the pathogenicity of the strains circulating in the wild (IV). Such data would be very important to the conservation of endangered species. The determinants of

AMDV pathogenicity (see Chapter 2.3.1. for references) are apparently complex and, in addition to host factors, interaction of several genes (Schuierer et al., 1997; McCrackin Stevenson et al., 2001) and other features (e.g., caspase cleavage and apoptosis) (Best et al., 2002; Cheng et al., 2010) are probably involved. In general, most field strains circulating at farms appear to be of low or moderate pathogenicity (Gorham et al., 1976; Hadlow et al., 1983; Porter, 1986). The limited clinicopathologic and epidemiologic data also suggest that several strains of variable pathogenicity are circulating within Finnish farms (Knuuttila, 2007). While certain studies show some degree of geographical clustering across countries or regions (Christensen et al., 2011; Nituch et al., 2012; Sang et al., 2012), others do not (study I and IV, Leimann et al., 2015).The lack of geographical clustering of Finnish strains may be due to largely unregulated movement of animals in the global mink trade, and the rather short duration of domestic eradication program. In Denmark, where the long-standing eradication has been quite successful in controlling AMDV, geographic clustering was more apparent (Christensen et al., 2011).

6.2.5. Host range in Finland (IV)

Anti-AMDV antibodies or viral DNA was detected in three of the eight species of free-ranging mustelids in Finland. Prevalence was highest in the feral American mink, followed by European badgers, and polecats. Samples from pine martens, Eurasian otters, least weasels, wolverine, and stoat were negative. The actual sample size (308) was only 20% smaller than the ideal sample size (385) suggested to provide an accurate estimate of prevalence in all mustelid species. Thus, the overall prevalence (13%, 95% CI 9–17) can be assumed to be quite reliable. However, it should be stressed that sample sizes for detecting disease or to estimate prevalence were too small for most species. As in the calculation of the sample size to assess prevalence, the estimated true prevalence was assumed to be 50% (which is often the case in feral mink) thus resulting in the largest required sample size. However, if an estimate of 5% to 40% (usually found in other species) would have been made, the required sample size would have been smaller, from 73 to 369. Thus, at least for pine martens, the sample size for estimating prevalence can be considered sufficient. Additionally, the 95% CI seemed to be acceptable for feral mink. Thus, although infection was identified in three species, it is possible that it is present in the other four species (maybe excluding the pine marten) and prevalence is higher or lower than reported here. More samples are required to resolve this issue. Especially, the infection status of the wolverine and polecat should be clarified as the wolverine is critically endangered and the polecat is considered vulnerable (Liukko et al., 2010). Aleutian mink disease virus is resistant to many environmental conditions (reviewed by Cho, 1976; Hussain et al., 2014), but it is possible that the storage conditions (at RT, 4 °C, and -20 °C) and time (up to several months) of the filter paper blood samples and the autolysis of some of the carcasses might have resulted in degradation of antibodies and/or viral DNA, thereby yielding false-negatives. Generally, regardless of some time and temperature dependent loss, filter paper sampling seems to be a robust and reliable method for serological and PCR assay (Prado et al., 2005; Liang et al., 2014; Dauner et al., 2015).

Aleutian mink disease virus infection, as far as is known, has not previously been reported in European badgers. In feral mink, similar or lower prevalences have been reported elsewhere in Europe (Mañas et al., 2001; Yamaguchi and Macdonald, 2001; Fournier-Chambrillon et al., 2004; Jensen et al., 2012). Compared to the farmed mink with a prevalence of 15% in 2014 (M. Eerola [Fin Furlab], personal communication 21.5.2015), the feral mink and badgers had a considerably higher prevalence. Aleutian mink disease virus-positive polecats, stoats, a European otter, and a pine marten have also been found previously (Mañas et al., 2001; Fournier-Chambrillon et al., 2004; Farid, 2013). Natural infections have not been studied in least weasels and wolverines. Experimentally, two wolverines have been inoculated with AMDV, but failed to develop antibodies or lesions (Kenyon et al., 1978). We detected a positive sample in a single Finnish polecat, whereas samples from Finnish otters, pine martens, least weasels, wolverine, and stoat were negative. However, we were able to obtain sequences from three Estonian pine martens. We also tested additional samples with negative results from captive mustelids: one sample from a wolverine (submitted by Helsinki Zoo [Korkeasaari] for necropsy in the Faculty of Veterinary Medicine) and 43 samples from least weasels (organs submitted by the University of Jyväskylä) (Knuuttila, 2014 unpublished observations). Blood samples from 23 Estonian raccoon dogs were analyzed due to their potential role in the transmission of AMDV, but these were also negative (Knuuttila, 2014 unpublished results, Leimann et al., 2015). These data were, however, excluded from study IV as the aim was to study Finnish free-ranging mustelids.

Although many species may become infected, disease in free-ranging mustelids has only been detected in mink (Cho and Greenfield, 1978; Mañas et al., 2001; Persson et al., 2015) and striped skunk (LaDouceur et al., 2014). Additionally, hypergammaglobulinemia, which is related to development of lesions in mink (Larsen and Porter, 1975; Henson et al., 1976; reviewed by Porter, 1986; reviewed by Jackson et al., 1996a), has been detected in a few European mink, stone martens, and one pine marten (Fournier-Chambrillon et al., 2004). The wild AMDV strains found in Finnish mink and badgers are probably not very pathogenic; although the prevalence was quite high in these species their population sizes are viable. However, pathogenicity of the putative new amdoparvovirus in Estonian badgers is puzzling. One of the recently discovered amdoparvoviruses was linked to diarrhea epidemics among farmed arctic fox and raccoon dog cubs (Shao et al., 2014). Less information is available on the other two new amdoparvoviruses in gray and red foxes (Li et al., 2011; Bodewes et al., 2014a) and their ability to cause disease. Thus, more data are needed on the pathogenesis and pathology of AMDV and other amdoparvoviruses in many of their host species, especially in those that are endangered.

Our results infer that some PCR methods may be less sensitive in detecting (and therefore less suitable for screening) AMDV infection in wild/feral animals. Many of the screening studies have used primers described either by Olofsson et al. (1999) (partial NS1) or Oie at al. (1996) (partial VP2). We noticed that these primers failed to identify all AMDV strains, as many seropositive samples were DNA negative with these methods. A similar tendency was noted in the confirmation testing of positive individuals in the ELISA test, which was initially performed with these primers (Aaltonen and Sironen unpublished observations). Three of seven positives were missed by the NS1 protocol and

seven of 18 with the VP2 protocol (study IV). As a result of this, a new pan-amdo qPCR test was developed based on the gray fox amdovirus sequence. Notably, the Estonian strains in study IV originated from the same samples used by Leimann et al. (2015). In this study, AMDV DNA was only identified in mink samples, whereas in study IV we also identified it in badgers, pine martens, and a polecat. At least in mink, the viremia may be transient and intermittent (Jensen et al., 2014), but in the case of highly pathogenic strains it seems to persist for at least 60 days (Oie et al., 1996). Regardless of that, we were able to detect AMDV DNA in the blood samples of all seropositive animals except the badger. In this species, none of the animals were determined to be positive by both ELISA and qPCR (5 ELISA-positive, but PCR-negative; 2 ELISA-negative, but PCR-positive). Given that the spleens of seropositive badgers were also negative for AMDV DNA, it is possible that they are able to clear the virus. Due to these findings we recommend using both serologic assays and PCR to screen for AMDV in free-ranging mustelids.

This was the first time that the AMDV VP2 ELISA was used for detecting anti-AMDV antibodies in species other than mink. Based on these results, it also seems suitable for this purpose. Except for the badgers, the qPCR and ELISA tests had only two discordant results; mink that were ELISA-negative but PCR-positive. This might be due to a recent infection with no or an undetectable level of antibodies. This study provided new information on the host range of AMDV in Finland and new detection methods were established that can detect a wide diversity of strains. These advancements will facilitate further research in control, conservation efforts, and biosecurity at farms and international borders.

6.2.6. AMDV has wide geographical distribution (IV)

Aleutian mink disease virus infection was distributed throughout most (10/17) of the sampled regions. The prevalence was quite high (>20%) in certain regions, but sample sizes were too small or the sampling was often biased towards infected or AMDV-free species (e.g., Lapland and pine marten) to reliably assess the regional prevalences and the 95% CIs were quite wide except for Kanta-Häme. However, the target sample size (5–60) necessary to detect disease was reached in 11 of 17 regions. Differences in prevalence between the regions were not statistically significant. Pooling regions into larger areas (e.g., the former provinces of Oulu, Lappi, Southern, Western, and Eastern Finland) and obtaining more observations (20–165/province) failed to reveal any significant differences in prevalence (Knuuttila, 2014 unpublished observations). Initially, we assumed that the prevalence would be higher in the western parts of Finland due to the long history of mink farming there. Interestingly, the prevalence was also quite high in eastern Finland. This might be due to suitable habitat for mink, intentional release of mink (Kauhala, 1996) and history of fur farming in Russia near the Finnish border. Further studies are necessary to clarify the prevalence of AMDV in each species across Finland and to determine whether any spatial differences exist.

6.2.7. Feral American mink and badgers have a higher odds for infection (IV)

While mink (OR 335), badger (OR 74) and the 2006–2009 sampling period (OR 5) were associated with AMDV infection in free-ranging mustelids, age, sex, region, or the presence of mink farm in the area (at the municipal or regional level) were not. We acknowledge that all potential risk factors were not studied here due to a limited number of observations. Additionally, different species were not studied separately, but they probably carry dissimilar risk factors due to differences in their ecology.

Significantly higher prevalence in feral American mink than in other species (Fournier-Chambrillon et al., 2004) and no difference between the sexes (Fournier-Chambrillon et al., 2004; Farid, 2013) has been reported by others. Similarly, an increased risk of infection has been reported in adult feral mink (Yamaguchi and Macdonald, 2001; Persson et al., 2015) and in adult and subadult free-ranging mustelids (Fournier-Chambrillon et al., 2004). In our study, the prevalence was higher in juveniles than in adults but the difference was not significant. The age was estimated subjectively by the sample collector, and this may have influenced results. Age could have been better assessed through an analysis of the cementum annuli in the teeth (Lyons et al., 2012). However, this would have led to reduced sample numbers or limited the number of teeth submitted for analysis due to a more laborious sampling protocol. Temporal fluctuations in AMDV prevalence in the wild has not been reported before. We tested whether the differences between years could have been caused by sampling infected regions or species during the earlier (2006–2009) and AMDV-free regions or species during the later (2010–2014) period. We observed that the higher prevalence from 2006–2009 was not due to a sampling bias (Knuuttila, 2014 unpublished observations). Whether this difference is incidental or due to other factors such as changes in population density or environmental conditions warrants further research. Furthermore, fluctuations in the population density of important prey animals such as voles, may also play a role and during high-density periods mustelids may breed intensively and spread the virus more effectively. The 2006–2009 sampling period coincided with a high-density phase of the vole population cycle (Korpela et al., 2013), whereas between 2010–2014 the numbers were more moderate. In Finnish farmed mink the prevalence was lower (3–11%) 2006–2009 and higher (11–15%) 2010–2014, contrary to what was found in free-ranging mustelids.

6.2.8. Transmission of AMDV in the wild (IV)

Although most of the strains in the wild and at farms are closely related and share common ancestors, results of the statistical and phylogenetic analyses (IV) suggest that mink farms do not represent a major source of AMDV infection in free-ranging populations of Finland, at least at the municipal or regional level. Infected animals were also detected in regions where no mink farms currently operate, such as Päijät-Häme, Etelä-Savo, and South Karelia (Figure 5). The AMDV prevalence in free-ranging mustelids was actually significantly lower in those regions that had mink farms (10.2%), in contrast to those regions that had no farms (21.9%). If the farms were providing a reservoir of infection for free-ranging mustelids, a greater prevalence would have been

expected in those regions with mink farms. The phylogeny also supported the notion of a relatively trivial role played by mink farms in AMDV transmission (Figure 6), where clusters were composed of strains from different regions. Furthermore, mixing of strains of farm and wild origin was not evident (except for a single case in Group IV) and the strains from farmed and feral mink appeared to form separate clusters. Conversely, these data infer that rather than farms, feral mink and possibly badgers seem to be more important in the transmission of AMDV in the wild. Those species that share territory or come into contact with feral mink may be at risk. Infected escapees from farms and infections acquired at farms and transmitted by wild/feral animals to farms may still play a local role, especially if biosecurity measures at farms are inadequate. A more detailed epidemiologic study taking into account the number of local farms, their size, infection status, contacts, and biosecurity protocols would reveal more data on the transmission of the virus in the vicinity of mink farms.

Until recently, data on the sylvatic cycle and transmission between farms and the wild have been scarce. Some studies suggest that the transmission cycles are separate (Leimann et al., 2015) and others infer an intersection (Oie et al., 1996; Nituch et al., 2011; Nituch et al., 2012; Nituch et al., 2015). This suggests that the situation varies between countries, and might reflect for instance the differences in the farming practices, density and number of farms, geography, size of the studied area, and populations of mustelid species and their ecology. As seen in study IV, strains isolated from wild and feral mink, although closely related to strains from farmed mink, tend to form separate clusters (Jensen et al., 2012; Nituch et al., 2012; Leimann et al., 2015). Also, Leimann et al. (2015) showed that although the strains in feral mink originate from the global mink trade, the current sylvatic and domestic transmission cycles in Estonian mink are separate. A Canadian study indicated that farms may act as a source of viral infections in the wild (Nituch et al., 2011). Interestingly, wild vicinal raccoons have been implicated in AD outbreaks in farms while transmission from raccoon-to-raccoon is unlikely (Oie et al., 1996). In the wild, striped skunks may transmit the virus to wild mink (Nituch et al., 2015). Furthermore, in Canada where mink is native, the virus is carried between free-ranging domestic, wild, and hybrid mink and between mink farms and wild mink (Nituch et al., 2012). Experimental studies further suggest that raccoon dogs, cats, dogs, ferrets, and mice may serve as AMDV reservoirs and may be involved in AMDV circulation in mink farms (Alexandersen et al., 1985b).

7. Conclusions

- A new recombinant AMDV VP2 antigen was developed. It spontaneously formed VLPs and was antigenic in several serologic methods. It can be produced quickly in large quantities and after simple purification works both in CIEP and ELISA.
- Based on this antigen, a new and accurate ELISA test for the serodiagnosis of AMDV infection in mink was developed, automated, and validated. This test has several appealing features including low cost, rapid processing of large batches, reduced labor requirement, and digital data management.
- A new, rapid, and simple blood sampling method, filter-paper 'blood comb', was developed for the ELISA test.
- The virus has been introduced at least three times to Finnish farms, but the exact origin of the Finnish strains remains to be clarified.
- Finnish AMDV strains showed high variability and strains from several genogroups are circulating in captive and wild populations.
- The phylogeny showed no clear clustering based on the year of isolation, geographical origin, host species, or pathogenicity.
- A new host species of AMDV, the European badger, was identified.
- In addition to the badger, AMDV infection was detected in the feral American mink and polecat in Finland. For the first time, AMDV sequence data was obtained from the polecat, pine marten, and badger.
- AMDV is widely distributed in the wild throughout Finland.
- Although Finnish free-ranging mustelids carry strains similar to mink (an indication of cross-species transmission), divergent strains representing potential novel amdoparvoviruses such as the one identified in Estonian badgers, may also be present.
- Factors associated with higher odds of AMDV infection in free-ranging mustelids were the species (feral American mink and badger) and sampling period (2006–2009).
- Domestic and sylvatic transmission cycles seem to be mainly separate, at least at the broad scale, and the major source of virus in the wild is feral mink and possibly badger. Mink farms, however, may have a greater impact at the local scale.
- These studies introduced new diagnostic tools that can be utilized in AMDV monitoring and eradication programs, as well as further research. Additional sequence data for investigating the molecular epidemiology of AMDV were generated. Details of AMDV epidemiology and phylogenetics in Finnish farmed mink and free-ranging mustelids were clarified, providing new information on AMDV strain diversity, host species, transmission, and factors associated with infection. These results may have an impact on best practice of biosecurity protocols, investigation of outbreaks at farms, and conservation of endangered mustelid species.

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References

Aasted, B., 1985. Aleutian disease of mink. Virology and immunology. Acta Pathol. Microbiol. Immunol. Scand. Suppl. 287, 1-47.

Aasted, B., Cohn, A., 1982. Inhibition of precipitation in counter current electrophoresis. A sensitive method for detection of mink antibodies to Aleutian disease virus. Acta Pathol. Microbiol. Immunol. Scand. [C] 90, 15-19.

Aasted, B., Bloom, M.E., 1984. Mink with Aleutian disease have high-affinity antiviral antibodies. Scand. J. Immunol. 19, 411-418.

Aasted, B., Leslie, R.G., 1991. Virus-specific B-lymphocytes are probably the primary targets for Aleutian disease virus. Vet. Immunol. Immunopathol. 28, 127-141.

Aasted, B., Avery, B., Cohn, A., 1984a. Serological analyses of different mink Aleutian disease virus strains. Arch. Virol. 80, 11-22.

Aasted, B., Tierney, G.S., Bloom, M.E., 1984b. Analysis of the quantity of antiviral antibodies from mink infected with different Aleutian disease virus strains. Scand. J. Immunol. 19, 395-402.

Aasted, B., Alexandersen, S., Christensen, J., 1998. 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 16, 1158-1165.

Aasted, B., Alexandersen, S., Cohn, A., Hansen, M., 1986. Counter current line absorption immunoelectrophoresis in an alternative diagnostic screening test to counter current immunoelectrophoresis in Aleutian disease (AD) eradication programs. Acta Vet. Scand. 27, 410-420.

Alexandersen, S., Bloom, M.E., Wolfinbarger, J., Race, R.E., 1987. *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. 61, 2407-2419.

Alexandersen, S., 1986. Acute interstitial pneumonia in mink kits: experimental reproduction of the disease. Vet. Pathol. 23, 579-588.

Alexandersen, S., 1990. Pathogenesis of disease caused by Aleutian mink disease parvovirus. APMIS Suppl. 14, 1-32.

Alexandersen, S., Hau, J., 1985. Rocket line immunoelectrophoresis: an improved assay for simultaneous quantification of a mink parvovirus (Aleutian disease virus) antigen and antibody. J. Virol. Methods 10, 145-151.

Alexandersen, S., Bloom, M.E., 1987. Studies on the sequential development of acute interstitial pneumonia caused by Aleutian disease virus in mink kits. J. Virol. 61, 81-86.

Alexandersen, S., Bloom, M.E., Wolfinbarger, J., 1988. Evidence of restricted viral replication in adult mink infected with Aleutian disease of mink parvovirus. J. Virol. 62, 1495-1507.

Alexandersen, S., Hau, J., Aasted, B., Poulsen, O.M., 1985a. Thin-layer counter current line absorption immunoelectrophoretic analysis of antigens and antibodies to Aleutian disease virus - a mink parvovirus. Electrophoresis 6, 535-538.

Alexandersen, S., Jensen, A.U., Hansen, M., Aasted, B., 1985b. Experimental transmission of Aleutian disease virus (ADV) to different animal species. Acta Pathol. Microbiol. Immunol. Scand. [B] 93, 195-200.

Alexandersen, S., Storgaard, T., Kamstrup, N., Aasted, B., Porter, D.D., 1994a. Pathogenesis of Aleutian mink disease parvovirus infection: effects of suppression of antibody response on viral mRNA levels and on development of acute disease. J. Virol. 68, 738-749.

Alexandersen, S., Larsen, S., Aasted, B., Uttenthal, A., Bloom, M.E., Hansen, M., 1994b. Acute interstitial pneumonia in mink kits inoculated with defined isolates of Aleutian mink disease parvovirus. Vet. Pathol. 31, 216-228.

Alexandersen, S., Larsen, S., Cohn, A., Uttenthal, A., Race, R.E., Aasted, B., Hansen, M., Bloom, M.E., 1989. Passive transfer of antiviral antibodies restricts replication of Aleutian mink disease parvovirus *in vivo*. J. Virol. 63, 9-17.

Andersson, A.M., Wallgren, P., 2013. Evaluation of two enzyme-linked immunosorbent assays for serodiagnosis of Aleutian mink disease virus infection in mink. Acta Vet. Scand. 55, 1-6.

Anistoroaei, R., Krogh, A.K., Christensen, K., 2013. A frameshift mutation in the LYST gene is responsible for the Aleutian color and the associated Chediak-Higashi syndrome in American mink. Anim. Genet. 44, 178-183.

Aronen, P., 2006. Working group for plasmacytosis control: how often should mink be tested? (In Finnish). Turkistalous 3, 36.

Best, S.M., Bloom, M.E., 2005. Pathogenesis of Aleutian mink disease parvovirus and similarities to B19 infection. J. Vet. Med. B Infect. Dis. Vet. Public Health 52, 331-334.

Best, S.M., Wolfinbarger, J.B., Bloom, M.E., 2002. Caspase activation is required for permissive replication of Aleutian mink disease parvovirus *in vitro*. Virology 292, 224-234.

BLAST, 2008. Basic Local Alignment Search Tool. http://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed January 2008.

BLAST, 2014. Basic Local Alignment Search Tool. http://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed July 2014.

Bloom, M.E., Alexandersen, S., Perryman, S., Lechner, D., Wolfinbarger, J.B., 1988. Nucleotide sequence and genomic organization of Aleutian mink disease parvovirus (ADV): sequence comparisons between a non-pathogenic and pathogenic strain of ADV. J. Virol. 62, 2903-2915.

Bloom, M.E., Alexandersen, S., Garon, C.F., Mori, S., Wei, W., Perryman, S., Wolfinbarger, J.B., 1990. Nucleotide sequence of the 5'-terminal palindrome of Aleutian mink disease parvovirus and construction of an infectious molecular clone. J. Virol. 64, 3551-3556.

Bloom, M.E., Race, R.E., Wolfinbarger, J.B., 1980. Characterization of Aleutian disease virus as a parvovirus. J. Virol. 35, 836-843.

Bloom, M.E., Race, R.E., Wolfinbarger, J.B., 1982. Identification of a nonvirion protein of Aleutian disease virus: mink with Aleutian disease have antibody to both virion and nonvirion proteins. J. Virol. 43, 608-616.

Bloom, M.E., Race, R.E., Hadlow, W.J., Chesebro, B., 1975. Aleutian disease of mink: the antibody response of sapphire and pastel mink to Aleutian disease virus. J. Immunol. 115, 1034-1037.

Bloom, M.E., Alexandersen, S., Mori, S., Wolfinbarger, J.B., 1989. Analysis of parvovirus infections using strand-specific hybridization probes. Virus Res. 14, 1-25.

Bloom, M.E., Kanno, H., Mori, S., Wolfinbarger, J.B., 1994. Aleutian mink disease: puzzles and paradigms. Infect. Agents Dis. 3, 279-301.

Bloom, M.E., Berry, B.D., Wei, W., Perryman, S., Wolfinbarger, J.B., 1993. Characterization of chimeric full-length molecular clones of Aleutian mink disease parvovirus (ADV): identification of a determinant governing replication of ADV in cell culture. J. Virol. 67, 5976-5988.

Bloom, M.E., Fox, J.M., Berry, B.D., Oie, K.L., Wolfinbarger, J.B., 1998. Construction of pathogenic molecular clones of Aleutian mink disease parvovirus that replicate both *in vivo* and *in vitro*. Virology 251, 288-296.

Bloom, M.E., Best, S.M., Hayes, S.F., Wells, R.D., Wolfinbarger, J.B., McKenna, R., Agbandje-McKenna, M., 2001. Identification of Aleutian mink disease parvovirus capsid sequences mediating antibody-dependent enhancement of infection, virus neutralization, and immune complex formation. J. Virol. 75, 11116-11127.

Bodewes, R., Ruiz-Gonzalez, A., Schapendonk, C.M., van den Brand, J.M., Osterhaus, A.D., Smits, S.L., 2014a. Viral metagenomic analysis of feces of wild small carnivores. Virol. J. 11, doi: 10.1186/1743-422X-11-89.

Bodewes, R., Lapp, S., Hahn, K., Habierski, A., Forster, C., Konig, M., Wohlsein, P., Osterhaus, A.D., Baumgartner, W., 2014b. Novel canine bocavirus strain associated with severe enteritis in a dog litter. Vet. Microbiol. 174, 1-8.

Bossuyt, P.M., Reitsma, J.B., Bruns, D.E., Gatsonis, C.A., Glasziou, P.P., Irwig, L.M., Lijmer, J.G., Moher, D., Rennie, D., de Vet, H.C., Standards for Reporting of Diagnostic Accuracy, 2003. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Standards for Reporting of Diagnostic Accuracy. Clin. Chem. 49, 1-6.

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T.G., Wu, C.-., Xie, D., Suchard, M.A., Rambaut, A., Drummond, A.J., 2014. BEAST2: A software platform for Bayesian evolutionary analysis. PLoS Comput Biol 10, doi: 10.1371/journal.pcbi.1003537.

Bowman, J., Kidd, A.G., Nituch, L.A., Sadowski, C., Schulte-Hostedde, A.I., 2014. Testing for Aleutian mink disease virus in the river otter (*Lontra canadensis*) in sympatry with infected American mink (*Neovison vison*). J. Wildl. Dis. 50, doi: 10.7589/2013-10-286.

Branscum, A.J., Gardner, I.A., Johnson, W.O., 2005. Estimation of diagnostic-test sensitivity and specificity through Bayesian modeling. Prev. Vet. Med. 68, 145-163.

Broll, S., Alexandersen, S., 1996. Investigation of the pathogenesis of transplacental transmission of Aleutian mink disease parvovirus in experimentally infected mink. J. Virol. 70, 1455-1466.

Brown, L.D., Cai, T.T., DasGupta, A., 2001. Interval estimation for a binomial proportion. Stat. Sci. 16, 101-133.

Castelruiz, Y., Blixenkrone-Moller, M., Aasted, B., 2005. DNA vaccination with the Aleutian mink disease virus NS1 gene confers partial protection against disease. Vaccine 23, 1225-1231.

Chapman, I., Jimenez, F.A., 1963. Aleutian-mink disease in man. N. Engl. J. Med. 269, 1171-1174.

Cheema, A., Henson, J.B., Gorham, J.R., 1972. Aleutian disease of mink. Prevention of lesions by immunosuppression. Am. J. Pathol. 66, 543-556.

Cheng, F., Chen, A.Y., Best, S.M., Bloom, M.E., Pintel, D., Qiu, J., 2010. The capsid proteins of Aleutian mink disease virus activate caspases and are specifically cleaved during infection. J. Virol. 84, 2687-2696.

Cho, H.J., 1976. Purification and structure of Aleutian disease virus. Front. Biol. 44, 159-174.

Cho, H.J., Ingram, D.G., 1972. Antigen and antibody in Aleutian disease in mink. I. Precipitation reaction by agar-gel electrophoresis. J. Immunol. 108, 555-557.

Cho, H.J., Ingram, D.G., 1974. The antigen and virus of Aleutian disease in mink. J. Immunol. Methods 4, 217-228.

Cho, H.J., Greenfield, J., 1978. Eradication of Aleutian disease of mink by eliminating positive counterimmunoelectrophoresis test reactors. J. Clin. Microbiol. 7, 18-22.

Christensen, J., Storgaard, T., Bloch, B., Alexandersen, S., Aasted, B., 1993. Expression of Aleutian mink disease parvovirus proteins in a baculovirus vector system. J. Virol. 67, 229-238.

Christensen, L.S., Gram-Hansen, L., Chriel, M., Jensen, T.H., 2011. Diversity and stability of Aleutian mink disease virus during bottleneck transitions resulting from eradication in domestic mink in Denmark. Vet. Microbiol. 149, 64-71.

Clemens, D.L., Wolfinbarger, J.B., Mori, S., Berry, B.D., Hayes, S.F., Bloom, M.E., 1992. Expression of Aleutian mink disease parvovirus capsid proteins by a recombinant vaccinia virus: self-assembly of capsid proteins into particles. J. Virol. 66, 3077-3085.

Costello, F., Steenfos, N., Jensen, K.T., Christensen, J., Gottschalck, E., Holm, A., Aasted, B., 1999. Epitope mapping of Aleutian mink disease parvovirus virion protein VP1 and 2. Scand. J. Immunol. 49, 347-354.

Cotmore, S.F., Agbandje-McKenna, M., Chiorini, J.A., Mukha, D.V., Pintel, D.J., Qiu, J., Söderlund-Venermo, M., Tattersall, P., Tijssen, P., Gatherer, D., Davison, A.J., 2014. The family Parvoviridae. Arch. Virol. 159, 1239-1247.

Crawford, T.B., McGuire, T.C., Porter, D.D., Cho, H.J., 1977. A comparative study of detection methods for Aleutian disease viral antibody. J. Immunol. 118, 1249-1251.

Curry, P.S., Elkin, B.T., Campbell, M., Nielsen, K., Hutchins, W., Ribble, C., Kutz, S.J., 2011. Filter-paper blood samples for ELISA detection of Brucella antibodies in caribou. J. Wildl. Dis. 47, 12-20.

da Costa, A.C., Bendit, I., de Oliveira, A.C., Kallas, E.G., Sabino, E.C., Sanabani, S.S., 2013. Investigation of human parvovirus B19 occurrence and genetic variability in different leukaemia entities. Clin. Microbiol. Infect. 19, doi: 10.1111/1469-0691.

Dam-Tuxen, R., Dahl, J., Jensen, T.H., Dam-Tuxen, T., Struve, T., Bruun, L., 2014. Diagnosing Aleutian mink disease infection by a new fully automated ELISA or by counter current immunoelectrophoresis: A comparison of sensitivity and specificity. J. Virol. Methods 199, 53-60.

Dauner, A.L., Gilliland, T.C.,Jr, Mitra, I., Pal, S., Morrison, A.C., Hontz, R.D., Wu, S.J., 2015. Evaluation of nucleic acid stabilization products for ambient temperature shipping and storage of viral RNA and antibody in a dried whole blood format. Am. J. Trop. Med. Hyg. , doi: 10.4269/ajtmh.15-0110.

Eklund, C.M., Hadlow, W.J., Kennedy, R.C., Boyle, C.C., Jackson, T.A., 1968. Aleutian disease of mink: properties of the etiologic agent and the host responses. J. Infect. Dis. 118, 510-526.

Espregueira Themudo, G., Houe, H., Agger, J.F., Ostergaard, J., Ersboll, A.K., 2012. Identification of biosecurity measures and spatial variables as potential risk factors for Aleutian disease in Danish mink farms. Prev. Vet. Med. 107, 134-141.

Eterpi, M., McDonnell, G., Thomas, V., 2009. Disinfection efficacy against parvoviruses compared with reference viruses. J. Hosp. Infect. 73, 64-70.

Farid, A.H., 2013. Aleutian mink disease virus in furbearing mammals in Nova Scotia, Canada. Acta Vet. Scand. 55, doi: 10.1186/1751-0147-55-10.

Farid, A.H., Segervall, J., 2014. A comparison between ELISA and CIEP for measuring antibody titres against Aleutian mink disease virus. Virol. & Mycol. 3, doi:10.4172/2161-0517.1000137.

Farid, A.H., Hussain, I., Arju, I., 2015. Detection of Aleutian mink disease virus DNA and antiviral antibodies in American mink (*Neovison vison*) 10 days postinoculation. J. Vet. Diagn. Invest. 27, 287-294.

Farid, A.H., Zillig, M.L., Finley, G.G., Smith, G.C., 2012. Prevalence of the Aleutian mink disease virus infection in Nova Scotia, Canada. Prev. Vet. Med. 106, 332-338.

Fournier-Chambrillon, C., Aasted, B., Perrot, A., Pontier, D., Sauvage, F., Artois, M., Cassiede, J.M., Chauby, X., Dal Molin, A., Simon, C., Fournier, P., 2004. Antibodies to Aleutian mink disease parvovirus in free-ranging European mink (*Mustela lutreola*) and other small carnivores from southwestern France. J. Wildl. Dis. 40, 394-402.

Fox, J.M., McCrackin Stevenson, M.A., Bloom, M.E., 1999. Replication of Aleutian mink disease parvovirus *in vivo* is influenced by residues in the VP2 protein. J. Virol. 73, 8713-8719.

Gardner, I.A., Nielsen, S.S., Whittington, R.J., Collins, M.T., Bakker, D., Harris, B., Sreevatsan, S., Lombard, J.E., Sweeney, R., Smith, D.R., Gavalchin, J., Eda, S., 2011. Consensus-based reporting standards for diagnostic test accuracy studies for paratuberculosis in ruminants. Prev. Vet. Med. 101, 18-34.

GenBank, 2008. National Institutions of Health, Genetic Sequence Database. www.ncbi.nlm.nih.gov/genbank/. Accessed January 2008.

GenBank, 2014. National Institutes of Health, Genetic Sequence Database. www.ncbi.nlm.nih.gov/genbank/. Accessed July 2014.

Georgiadis, M.P., Johnson, W.O., Gardner, I.A., Singh, R., 2003. Correlation-adjusted estimation of sensitivity and specificity of two diagnostic tests. J. R. Stat. Soc. Ser. C Appl. Stat. 52, 63-76.

Gorham, J.R., Leader, R.W., Henson, J.B., 1964. The experimental transmission of a virus causing hypergammaglobulinemia in mink: sources and modes of infection. J. Infect. Dis. 114, 341-345.

Gorham, J.R., Henson, J.B., Crawford, T.B., Padgett, G.A., 1976. The epizootiology of Aleutian disease. Front. Biol. 44, 135-158.

Gottschalck, E., Alexandersen, S., Storgaard, T., Bloom, M.E., Aasted, B., 1994. Sequence comparison of the non-structural genes of four different types of Aleutian mink disease parvovirus indicates an unusual degree of variability. Arch. Virol. 138, 213-231.

Gottschalck, E., Alexandersen, S., Cohn, A., Poulsen, L.A., Bloom, M.E., Aasted, B., 1991. Nucleotide sequence analysis of Aleutian mink disease parvovirus shows that multiple virus types are present in infected mink. J. Virol. 65, 4378-4386.

Greiner, M., Gardner, I.A., 2000. Epidemiologic issues in the validation of veterinary diagnostic tests. Prev. Vet. Med. 45, 3-22.

Gunnarsson, E., 2001. Documenting freedom from disease and re-establishing a free status after a breakdown Aleutian disease (plasmacytosis) in farmed mink in Iceland. Acta Vet. Scand. 42, doi:10.1186/1751-0147-42-S1-S87.

Guthrie, R., Susi, A., 1963. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. Pediatrics 32, 338-343.

Haas, L., Wohlsein, P., Trautwein, G., Stolze, B., Kaaden, O.R., 1990. Violet mink develop an acute disease after experimental infection with Aleutian disease virus (ADV) isolate ADV SL3. Zentralbl. Veterinarmed. B. 37, 106-117.

Hadlow, W.J., 1982. Ocular lesions in mink affected with Aleutian disease. Vet. Pathol. 19, 5-15.

Hadlow, W.J., Race, R.E., Kennedy, R.C., 1983. Comparative pathogenicity of four strains of Aleutian disease virus for pastel and sapphire mink. Infect. Immun. 41, 1016-1023.

Hadlow, W.J., Race, R.E., Kennedy, R.C., 1984. Royal pastel mink respond variously to inoculation with Aleutian disease virus of low virulence. J. Virol. 50, 38-41.

Hadlow, W.J., Race, R.E., Kennedy, R.C., 1985. Temporal replication of the Pullman strain of Aleutian disease virus in royal pastel mink. J. Virol. 55, 853-856.

Hahn, E.C., Hahn, P.S., 1983. Autoimmunity in Aleutian disease: contribution of antiviral and anti-DNA antibody to hypergammaglobulinemia. Infect. Immun. 41, 494-500.

Häkli, K., 2013. Genetic parameters of minks Aleutian disease infection and fertility traits. (In Finnish with an English abstract). Master's Thesis. Faculty of Agriculture and Forestry, University of Helsinki, Finland.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41, 95-98.

Hammer, A.S., Dietz, H.H., Hamilton-Dutoit, S., 2007. Immunohistochemical detection of 3 viral infections in paraffin-embedded tissue from mink (*Mustela vison*): a tissue-microarray-based study. Can. J. Vet. Res. 71, 8-13.

Hartsough, G.R., Gorham, J.R., 1956. Aleutian disease in mink. Natl. Fur News 28, 10-11.

Hasegawa, M., Kishino, H., Yano, T., 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160-174.

Helmboldt, C.F., Kenyon, A.J., Dessel, B.H., 1965. The comparative aspects of Aleutian disease (AD). In: Gajdusek, D.C., Gibbs, C.J., Alpers, M.P. (Eds.), Slow, latent, and temperate virus infections. US Department of Health, Education and Welfare, Washington, pp. 315-319.

Henson, J.B., Gorham, J.R., McGuire, T.C., Crawford, T.B., 1976. Pathology and pathogenesis of Aleutian disease. Front. Biol. 44, 175-205.

Henttonen, H., 1992. The European mink (In Finnish). In: Koivisto, I. (Ed.), Endangered species of the world, 3: Finland. Weilin+Göös, pp. 46-48.

Hillyer, E.V., Brown, S.A., 2000. Ferrets. In: Birchard, S.J., Sherding, R.G. (Eds.), Saunders manual of small animal practice, 2nd ed. Saunders, Philadelphia, pp. 1464-1492.

Huang, Q., Deng, X., Best, S.M., Bloom, M.E., Li, Y., Qiu, J., 2012. Internal polyadenylation of parvoviral precursor mRNA limits progeny virus production. Virology 426, 167-177.

Huang, Q., Luo, Y., Cheng, F., Best, S.M., Bloom, M.E., Qiu, J., 2014. Molecular characterization of the small nonstructural proteins of parvovirus Aleutian mink disease virus (AMDV) during infection. Virology 452-453, 23-31.

Huson, D.H., Bryant, D., 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23, 254-267.

Hussain, I., Price, G.W., Farid, A.H., 2014. Inactivation of Aleutian mink disease virus through high temperature exposure *in vitro* and under field-based composting conditions. Vet. Microbiol. 173, 50-58.

Hyllseth, B., Nordstoga, K., Loftsgaard, G., Leathers, C.W., Gorham, J.R., 1992. Different pathogenicities of two Aleutian disease virus (ADV) strains in Norway. Norw. J. Agri. Sci. Suppl. 9, 459-468.

ICTV 2014. International Committee on Taxonomy of Viruses. Taxonomy 2014 Release. www.ictvonline.org/virusTaxonomy.asp. Accessed July 2014.

Ingram, D.G., Cho, H.J., 1974. Aleutian disease in mink: virology, immunology and pathogenesis. J. Rheumatol. 1, 74-92.

IUCN 2014. The International Union for Conservation of Nature red list of threatened species. www.iucnredlist.org/amazing-species. Accessed July 2014.

Jackson, M.K., Ellis, L.C., Morrey, J.D., Li, Z.Z., Barnard, D.L., 1996a. Progression of Aleutian disease in natural and experimentally induced infections of mink. Am. J. Vet. Res. 57, 1753-1758.

Jackson, M.K., Winslow, S.G., Dockery, L.D., Jones, J.K., Sisson, D.V., 1996b. Investigation of an outbreak of Aleutian disease on a commercial mink ranch. Am. J. Vet. Res. 57, 1706-1710.

Jahns, H., Daly, P., McElroy, M.C., Sammin, D.J., Bassett, H.F., Callanan, J.J., 2010. Neuropathologic features of Aleutian disease in farmed mink in Ireland and molecular characterization of Aleutian mink disease virus detected in brain tissues. J. Vet. Diagn. Invest. 22, 101-105.

Jensen, T.H., Hammer, A.S., Chriel, M., 2014. Monitoring chronic infection with a field strain of Aleutian mink disease virus. Vet. Microbiol. 168, 420-427.

Jensen, T.H., Christensen, L.S., Chriel, M., Uttenthal, A., Hammer, A.S., 2011. Implementation and validation of a sensitive PCR detection method in the eradication campaign against Aleutian mink disease virus. J. Virol. Methods 171, 81-85.

Jensen, T.H., Christensen, L.S., Chriél, M., Harslund, J., Salomonsen, C.M., Hammer, A.S., 2012. High prevalence of Aleutian mink disease virus in free-ranging mink on a remote Danish island. J. Wildl. Dis. 48, 497-502.

Jepsen, J.R., d'Amore, F., Baandrup, U., Clausen, M.R., Gottschalck, E., Aasted, B., 2009. Aleutian mink disease virus and humans. Emerg. Infect. Dis. 15, 2040-2042.

Kangas, J., Smeds, E., 1980s. Plasmacytosis in mink and its control measures. Studies on fur animals N:o 10. (In Finnish). Finnish Fur Breeders' Association, Vaasa, Finland.

Kankkonen, J., 2006. Working group for plasmacytosis control: control measures for plasmacytosis at Hirvlax. (In Finnish). Turkistalous 5, 13-15.

Kanno, H., Wolfinbarger, J.B., Bloom, M.E., 1992. Identification of Aleutian mink disease parvovirus transcripts in macrophages of infected adult mink. J. Virol. 66, 5305-5312.

Kanno, H., Wolfinbarger, J.B., Bloom, M.E., 1993. Aleutian mink disease parvovirus infection of mink macrophages and human macrophage cell line U937: demonstration of antibody-dependent enhancement of infection. J. Virol. 67, 7017-7024.

Karstad, L., Pridham, T.J., 1962. Aleutian disease of mink: I. Evidence of its viral etiology. Can. J. Comp. Med. Vet. Sci. 26, 97-102.

Kauhala, K., 1996. Distributional history of the American mink (*Mustela vison*) in Finland with special reference to the trends in otter (*Lutra lutra*) populations. Ann. Zool. Fennici 33, 283-291.

Kenyon, A.J., Kenyon, B.J., Hahn, E.C., 1978. Protides of the Mustelidae: immunoresponse of mustelids to Aleutian mink disease virus. Am. J. Vet. Res. 39, 1011-1015.

Knuuttila, A., 2007. Phylogenetic analysis of Aleutian mink disease parvovirus strains. Licentiate thesis. Faculty of Veterinary Medicine, University of Helsinki, Finland.

Kontou, M., Govindasamy, L., Nam, H.J., Bryant, N., Llamas-Saiz, A.L., Foces-Foces, C., Hernando, E., Rubio, M.P., McKenna, R., Almendral, J.M., Agbandje-McKenna, M., 2005. Structural determinants of tissue tropism and *in vivo* pathogenicity for the parvovirus minute virus of mice. J. Virol. 79, 10931-10943.

Korber, B., 2000. HIV signature and sequence variation analysis. In: Rodrigo, A.G., Learn, G.H. (Eds.), Computational analysis of HIV molecular sequences. Kluwer Academic Publishers, Dordrecht, Netherlands, pp. 55-72.

Korpela, K., Delgado, M., Henttonen, H., Korpimäki, E., Koskela, E., Ovaskainen, O., Pietiäinen, H., Sundell, J., Yoccoz, N., Huitu, O., 2013. Non-linear effects of climate on boreal rodent dynamics: warm winters do not negate high-amplitude cycles. Global Change Biology 13, 697-710.

Kryazhimskiy, S., Plotkin, J.B., 2008. The population genetics of dN/dS. PLoS Genet. 4, doi: 10.1371/journal.pgen.1000304.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief. Bioinform. 5, 150-163.

LaDouceur, E.E., Anderson, M., Ritchie, B.W., Ciembor, P., Rimoldi, G., Piazza, M., Pesti, D., Clifford, D.L., Giannitti, F., 2014. Aleutian disease: An emerging disease in free-ranging striped skunks (*Mephitis mephitis*) from California. Vet. Pathol. pii: 0300985814560234. [Epub ahead of print].

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Larivère, S., Jennings, A.P., 2009. Family Mustelidae (weasels and relatives). In: Wilson, D.E., Mittermeier, R.A. (Eds.), Handbook of the mammals of the world. Volume 1. Carnivores. Lynx Edicions, Barcelona, pp. 564-656.

Larsen, A.E., Porter, D.D., 1975. Pathogenesis of Aleutian disease of mink: identification of nonpersistent infections. Infect. Immun. 11, 92-94.

Leimann, A., Knuuttila, A., Maran, T., Vapalahti, O., Saarma, U., 2015. Molecular epidemiology of Aleutian mink disease virus (AMDV) in Estonia, and a global phylogeny of AMDV. Virus Res. 199, 56-61.

Li, L., Pesavento, P.A., Woods, L., Clifford, D.L., Luff, J., Wang, C., Delwart, E., 2011. Novel amdovirus in gray foxes. Emerg. Infect. Dis. 17, 1876-1878.

Li, Y., Huang, J., Jia, Y., Du, Y., Jiang, P., Zhang, R., 2012. Genetic characterization of Aleutian mink disease viruses isolated in China. Virus Genes 45, 24-30.

Liang, X., Chigerwe, M., Hietala, S.K., Crossley, B.M., 2014. Evaluation of Fast Technology Analysis (FTA) Cards as an improved method for specimen collection and shipment targeting viruses associated with Bovine Respiratory Disease Complex. J. Virol. Methods 202, 69-72.

Liukko, U.-M., Henttonen, H., Hanski, I.K., Kauhala, K., Kojola, I., Kyheröinen, E.-M., 2010. Mammals. In: Rassi, P., Hyvärinen, E., Juslén, A. et al (Eds.), The 2010 Red list of Finnish species. Ministry of the Environment and Finnish Environment Institute, pp. 311-319.

Lukashov, V.V., Goudsmit, J., 2001. Evolutionary relationships among parvoviruses: virus-host coevolution among autonomous primate parvoviruses and links between adeno-associated and avian parvoviruses. J. Virol. 75, 2729-2740.

Luke 2013. The Natural Resources Institute Finland. Small game (in Finnish). www.luke.fi. Accessed: May 2015.

Lund, A., 1979. The extent and development of mink breeding (in Finnish). In: Lund, A., Rochmann, R., Sanne, S. et al (Eds.), Breeding of mink - Nordic handbook for mink breeders. Dansk Pelsdyravlerforening, Norges Pelsdyralslag, Suomen Turkiseläinten Kasvattajain Liitto, Sveriges Pälsdjursuppfödares Riksförbund, Jyväskylä, pp. 9-13.

Lunn, D.J., Thomas, A., Best, N., Spiegelhalter, D., 2000. WinBUGS - A Bayesian modelling framework: concepts, structure, and extensibility. Stat. Comput. 10, 325-337.

Lyons, E.K., Schroeder, M.A., Robb, L.A., 2012. Criteria for determening sex and age of birds and mammals. In: Silvy, N.J. (Ed.), The wildlife techniques manual: Volume 1: Research. The Johns Hopkins University Press, Baltimore, pp. 207-229.

MacLachlan, N.J., Dubovi, E.J., 2011. Fenner's Veterinary Virology, 4th ed. Academic Press Elsevier, London, UK.

Mañas, S., Cena, J.C., Ruiz-Olmo, J., Palazon, S., Domingo, M., Wolfinbarger, J.B., Bloom, M.E., 2001. Aleutian mink disease parvovirus in wild riparian carnivores in Spain. J. Wildl. Dis. 37, 138-144.

Maran, T., Henttonen, H., 1995. Why is the European mink (Mustela lutreola) disappearing? - A review of the process and hypotheses. Ann. Zool. Fennici 32, 47-54.

Matsuura, Y., Possee, R.D., Overton, H.A., Bishop, D.H., 1987. Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. J. Gen. Virol. 68 (Pt 5), 1233-1250.

McCrackin Stevenson, M.A., Fox, J.M., Wolfinbarger, J.B., Bloom, M.E., 2001. Effect of a valine residue at codon 352 of the VP2 capsid protein on *in vivo* replication and pathogenesis of Aleutian disease parvovirus in mink. Am. J. Vet. Res. 62, 1658-1663.

McGuire, T.C., Crawford, T.B., 1980. Antibodies to Aleutian disease virus in human sera. J. Infect. Dis. 142, 625.

McKenna, R., Olson, N.H., Chipman, P.R., Baker, T.S., Booth, T.F., Christensen, J., Aasted, B., Fox, J.M., Bloom, M.E., Wolfinbarger, J.B., Agbandje-McKenna, M., 1999. Three-dimensional structure of Aleutian mink disease parvovirus: implications for disease pathogenicity. J. Virol. 73, 6882-6891.

Mei, J.V., Alexander, J.R., Adam, B.W., Hannon, W.H., 2001. Use of filter paper for the collection and analysis of human whole blood specimens. J. Nutr. 131, 1631S-6S.

Miroshnichenko, S.M., Peremislov, V.V., Taranin, A.V., 1992. Enzyme immunoassay of antibodies against Aleutian disease virus. Norw. J. Agri. Sci. Suppl. 9, 388-392.

Mori, S., Wolfinbarger, J.B., Miyazawa, M., Bloom, M.E., 1991. Replication of Aleutian mink disease parvovirus in lymphoid tissues of adult mink: involvement of follicular dendritic cells and macrophages. J. Virol. 65, 952-956.

Murakami, M., Matsuba, C., Une, Y., Nomura, Y., Fujitani, H., 2001. Nucleotide sequence and polymerase chain reaction/restriction fragment length polymorphism analyses of Aleutian disease virus in ferrets in Japan. J. Vet. Diagn. Invest. 13, 337-340.

Nei, M., Gojobori, T., 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. 3, 418-426.

Nes, N.N., Einarsson, E.J., Lohi, O., Jorgensen, G., 1988. Beautiful fur animals - and their colour genetics. Scientifur, Hillerod, Denmark.

Ni, J., Qiao, C., Han, X., Han, T., Kang, W., Zi, Z., Cao, Z., Zhai, X., Cai, X., 2014. Identification and genomic characterization of a novel porcine parvovirus (PPV6) in China. Virol. J. 11, 203.

Nicholas, K.B. and Nicholas, H.B.Jr. 1997. Genedoc: a tool for editing and annotating multiple sequence alignments. www.psc.edu/biomed/genedoc. Accessed January 2008.

Nituch, L.A., Bowman, J., Beauclerc, K.B., Schulte-Hostedde, A.I., 2011. Mink farms predict Aleutian disease exposure in wild American mink. PLoS One 6, doi: 10.1371/journal.pone.0021693.

Nituch, L.A., Bowman, J., Wilson, P., Schulte-Hostedde, A.I., 2012. Molecular epidemiology of Aleutian disease virus in free-ranging domestic, hybrid, and wild mink. Evol. Appl. 5, 330-340.

Nituch, L.A., Bowman, J., Wilson, P.J., Schulte-Hostedde, A.I., 2015. Aleutian mink disease virus in striped skunks (*Mephitis mephitis*): evidence for cross-species spillover. J. Wildl. Dis. 51, 389-400.

Nuesch, J.P., Rommelaere, J., 2014. Tumor suppressing properties of rodent parvovirus NS1 proteins and their derivatives. Adv. Exp. Med. Biol. 818, 99-124.

OIE, 2008. The World Organisation for Animal Health. Manual of diagnostic tests and vaccines for terrestrial animals. 6th ed., vol. 1. Chapter 1.1.4. Principles of validation of diagnostic assays for infectious diseases. www.oie.int/doc/ged/D7710.pdf. Accessed January 2008.

OIE 2013. The World Organisation for Animal Health. Manual of diagnostic tests and vaccines for terrestrial animals. Chapter 1.1.5. Principles and methods of validation of diagnostic assays for infectious diseases. www.oie.int/fileadmin/Home/eng/Health_standards/tahm/1.01.05_VALIDATION.pdf. Accessed March 2014.

OIE 2014. The World Organisation for Animal Health. Validation guideline 3.6.5. Statistical approaches to validation. www.oie.int/fileadmin/Home/eng/Health_standards/tahm/GUIDELINE_3.6.5_STATISTICAL_VALIDATION.pdf. Accessed May 2015.

OIE 2015. The World Organisation for Animal Health. OIE-listed diseases, infections and infestations. www.oie.int/animal-health-in-the-world/oie-listed-diseases-2015/. Accessed May 2015.

Oie, K.L., Durrant, G., Wolfinbarger, J.B., Martin, D., Costello, F., Perryman, S., Hogan, D., Hadlow, W.J., Bloom, M.E., 1996. 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. 70, 852-861.

Olofsson, A., Mittelholzer, C., Treiberg Berndtsson, L., Lind, L., Mejerland, T., Belak, S., 1999. Unusual, high genetic diversity of Aleutian mink disease virus. J. Clin. Microbiol. 37, 4145-4149.

Padgett, G.A., Gorham, J.R., Henson, J.B., 1967. Epizootiologic studies of Aleutian disease. I. Transplacental transmission of the virus. J. Infect. Dis. 117, 35-38.

Parrish, C.R., 2011. Parvoviridae. In: MacLachlan, N.J., Dubovi, E.J. (Eds.), Fenner's Veterinary Virology, 4th ed. Academic Press Elsevier, London, pp. 225-235.

Persson, S., Jensen, T.H., Blomstrom, A.L., Appelberg, M.T., Magnusson, U., 2015. Aleutian mink disease virus in free-ranging mink from Sweden. PLoS One 10, doi: 10.1371/journal.pone.0122194.

Poikulainen, E., 2014. Productization of ELISA test for Aleutian mink disease. (In Finnish with an English abstract). Thesis, Helsinki Metropolia University of Applied Sciences, Finland.

Porter, D.D., 1986. Aleutian disease: a persistent parvovirus infection of mink with a maximal but ineffective host humoral immune response. Prog. Med. Virol. 33, 42-60.

Porter, D.D., Larsen, A.E., Porter, H.G., 1969. The pathogenesis of Aleutian disease of mink. I. *In vivo* viral replication and the host antibody response to viral antigen. J. Exp. Med. 130, 575-593.

Porter, D.D., Larsen, A.E., Porter, H.G., 1972. 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. 109, 1-7.

Porter, D.D., Larsen, A.E., Porter, H.G., 1977a. Reduced severity of lesions in mink infected transplacentally with Aleutian disease virus. J. Immunol. 119, 872-876.

Porter, D.D., Larsen, A.E., Porter, H.G., 1980. Aleutian disease of mink. Adv. Immunol. 29, 261-286.

Porter, D.D., Porter, H.G., Larsen, A.E., 1984a. Much of the increased IgG in Aleutian disease of mink is viral antibody. J. Exp. Pathol. 1, 79-88.

Porter, D.D., Porter, H.G., Suffin, S.C., Larsen, A.E., 1984b. Immunoglobulin classes of Aleutian disease virus antibody. Infect. Immun. 43, 463-466.

Porter, D.D., Larsen, A.E., Cox, N.A., Porter, H.G., Suffin, S.C., 1977b. Isolation of Aleutian disease virus of mink in cell culture. Intervirology 8, 129-144.

Porter, H.G., Porter, D.D., Larsen, A.E., 1982. Aleutian disease in ferrets. Infect. Immun. 36, 379-386.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817-818.

Prado, I., Rosario, D., Bernardo, L., Alvarez, M., Rodriguez, R., Vazquez, S., Guzman, M.G., 2005. PCR detection of dengue virus using dried whole blood spotted on filter paper. J. Virol. Methods 125, 75-81.

Prieto, A., Diaz-Cao, J.M., Fernandez-Antonio, R., Panadero, R., Diaz, P., Lopez, C., Morrondo, P., Diez-Banos, P., Fernandez, G., 2014. Application of real-time PCR to detect Aleutian mink disease virus on environmental farm sources. Vet. Microbiol. 173, 355-359.

Profur, 2014. Statistics (in Finnish). Finnish Fur Breeders' Association, Vantaa, Finland.

Qiu, J., Cheng, F., Burger, L.R., Pintel, D., 2006. The transcription profile Aleutian mink disease virus in CRFK cells is generated by alternative processing of pre-mRNAs produced from a single promoter. J. Virol. 80, 654-662.

Qiu, J., Cheng, F., Pintel, D., 2007. The abundant R2 mRNA generated by the Aleutian mink disease parvovirus is tri-cistronic, encoding NS2, VP1, and VP2. J. Virol. 81, 6993-7000.

Reid, F., Helgen, K., 2008. Neovison vison. www.iucnredlist.org. Accessed May 2015.

Saifuddin, M., Fox, J.G., 1996. Identification of a DNA segment in ferret Aleutian disease virus similar to a hypervariable capsid region of mink Aleutian disease parvovirus. Arch. Virol. 141, 1329-1336.

Sanchez-Migallon Guzman, D., Carvajal, A., Garcia-Marin, J.F., Ferreras, M.C., Perez, V., Mitchell, M., Urra, F., Cena, J.C., 2008. Aleutian disease serology, protein electrophoresis, and pathology of the European mink (*Mustela lutreola*) from Navarra, Spain. J. Zoo Wildl. Med. 39, 305-313.

Sang, Y., Ma, J., Hou, Z., Zhang, Y., 2012. Phylogenetic analysis of the VP2 gene of Aleutian mink disease parvoviruses isolated from 2009 to 2011 in China. Virus Genes 45, 31-37.

Schmidt, H.A., Strimmer, K., Vingron, M., von Haeseler, A., 2002. TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics 18, 502-504.

Schuierer, S., Bloom, M.E., Kaaden, O.R., Truyen, U., 1997. Sequence analysis of the lymphotropic Aleutian disease parvovirus ADV-SL3. Arch. Virol. 142, 157-166.

Sergeant, E.S.G., 2013. Epitools epidemiological calculators. AusVet Animal Health Services and Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease. http://epitools.ausvet.com.au. Accessed June 2013, March 2014, July 2015, and May 2015.

Shackelford, R.M., 1957. Genetics of the ranch mink. Pilsbury Publishers, Inc., New York.

Shackelton, L.A., Hoelzer, K., Parrish, C.R., Holmes, E.C., 2007. Comparative analysis reveals frequent recombination in the parvoviruses. J. Gen. Virol. 88, 3294-3301.

Shao, X.Q., Wen, Y.J., Ba, H.X., Zhang, X.T., Yue, Z.G., Wang, K.J., Li, C.Y., Qiu, J., Yang, F.H., 2014. Novel amdoparvovirus infecting farmed raccoon dogs and arctic foxes. Emerg. Infect. Dis. 20, 2085-2088.

Sico, C., White, S., Tsao, E., Varma, A., 2002. Enhanced kinetic extraction of parvovirus B19 structural proteins. Biotechnol. Bioeng. 80, 250-256.

Söderlund-Venermo, M., Hokynar, K., Nieminen, J., Rautakorpi, H., Hedman, K., 2002. Persistence of human parvovirus B19 in human tissues. Pathol. Biol. (Paris) 50, 307-316.

STKL 2015a. Finnish Fur Breeders' Association. Basic information of fur farms (in Finnish). www.turkistieto.fi. Accessed May 2015.

STKL 2015b. Finnish Fur Breeders' Association. How often to test (in Finnish). www.profur.fi/Kuinka_usein_testataan. Accessed May 2015.

Swofford, D.L., 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Sinauer Associates, Sunderland, Massachusetts, USA version 4.

SYKE 2014. Finnish Environment Institute. Information on species: species of the Habitats Directive (in Finnish). www.ymparisto.fi/lajiesittelyt. Accessed May 2015.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731-2739.

Tewary, S.K., Zhao, H., Deng, X., Qiu, J., Tang, L., 2014. The human parvovirus B19 non-structural protein 1 N-terminal domain specifically binds to the origin of replication in the viral DNA. Virology 449, 297-303.

Themudo, G.E., Østergaard, J., Ersbøll, A.K., 2011. Persistent spatial clusters of plasmacytosis among Danish mink farms. Prev. Vet. Med. 102, 75-82.

Thermo Fisher Scientific, 2014. DNA Copy Number Calculator. www.thermoscientificbio.com/webtools/copynumber/. Accessed July 2014.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22, 4673-4680.

Thrusfield, M., 2007a. The control and eradication of disease. Veterinary epidemiology, 3rd ed. Blackwell Publishing, Oxford, pp. 384-403.

Thrusfield, M., 2007b. Diagnostic testing. Veterinary epidemiology, 3rd ed. Blackwell Publishing, Oxford, pp. 305-330.

Tijssen, P., Agbandje-McKenna, M., Almendral, J.M., Bergoin, M., Flegel, T.W., Hedman, K., Kleinschmidt, J., Li, Y., Pintel, D.J., Tattersall, P., 2011. Family - Parvoviridae. In: King, A.M.Q., Adams, M.J., Carstens, E.B. et al (Eds.), Virus taxonomy. Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, London, pp. 405-425.

Trautwein, G.W., Helmboldt, C.F., 1962. Aleutian disease of mink. I. Experimental transmission of the disease. Am. J. Vet. Res. 23, 1280-1288.

Uttenthal, A., 1992. Screening for antibodies against Aleutian disease virus (ADV) in mink. Elucidation of dubious results by additive counterimmunoelectrophoresis. Appl. Theor. Electrophor. 3, 83-84.

van Dawen, S., Kaaden, O.R., Roth, S., 1983. Propagation of Aleutian disease parvovirus in cell line CCC clone 81. Arch. Virol. 77, 39-50.

Wang, J., Cheng, S., Yi, L., Cheng, Y., Yang, S., Xu, H., Zhao, H., Yan, X., Wu, H., 2012. Evidence for natural recombination between mink enteritis virus and canine parvovirus. Virol. J. 9, doi: 10.1186/1743-422X-9-252.

Welchman Dde, B., Oxenham, M., Done, S.H., 1993. Aleutian disease in domestic ferrets: diagnostic findings and survey results. Vet. Rec. 132, 479-484.

Westgard, J., 2009. Westgard QC. www.westgard.com. Accessed July 2015.

Westman, K., 1966. Occurence of feral American minks (*Mustela vison*) in Finland (in Finnish). Suomen Riista 18, 101-116.

Wohlsein, P., Trautwein, G., Stolze, B., Haas, L., Kaaden, O.R., 1990. Antigen distribution in organs of mink with Aleutian disease parvovirus infection. Zentralbl. Veterinarmed. B. 37, 651-659.

Wright, P.F., Wilkie, B.N., 1982. Detection of antibody in Aleutian disease of mink: comparison of enzyme-linked immunosorbent assay and counterimmunoelectrophoresis. Am. J. Vet. Res. 43, 865-868.

Wu, W.H., Bloom, M.E., Berry, B.D., McGinley, M.J., Platt, K.B., 1994. Expression of Aleutian mink disease parvovirus capsid proteins in a baculovirus expression system for potential diagnostic use. J. Vet. Diagn. Invest. 6, 23-29.

Yahiro, T., Wangchuk, S., Tshering, K., Bandhari, P., Zangmo, S., Dorji, T., Tshering, K., Matsumoto, T., Nishizono, A., Söderlund-Venermo, M., Ahmed, K., 2014. Novel human bufavirus genotype 3 in children with severe diarrhea, Bhutan. Emerg. Infect. Dis. 20, 1037-1039.

Yamaguchi, N., Macdonald, D.W., 2001. Detection of Aleutian disease antibodies in feral American mink in southern England. Vet. Rec. 149, 485-488.

Zeng, X.W., Hua, Y.P., Liang, D.Y., 2007. Prokaryotic expression and detective application of the main antigenic region of VP2 protein of Aleutian mink disease parvovirus (in Chinese with an English abstract). Wei Sheng Wu Xue Bao 47, 1088-1090.

Appendix 1

Aleutian mink disease virus strains studied in this thesis

Strain	Species	Country, region	Isolation year	Pathogenicity	Reference	GenBank acc. no.
FIN05/B4[a]	Farmed American mink	Finland, Ostrobothnia	2005	ND[b]	I and IV	EU908029 and KM374785
FIN05/B5	-‘’-	-‘’-	-‘’-	-‘’-	I	EU908030
FIN05/B6	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	EU908031
FIN05/C7	-‘’-	-‘’-	-‘’-	-‘’-	I and IV	EU908032 and KM374782
FIN05/C8	-‘’-	-‘’-	-‘’-	-‘’-	I and II	EU908033 and GQ336866
FIN05/C9	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	EU908034
FIN05/D10	-‘’-	Finland, North Ostrobothnia	-‘’-	high	I and IV	EU908035 and KM374783
FIN05/D11	-‘’-	-‘’-	-‘’-	-‘’-	I	EU908036
FIN05/D12	-‘’-	-‘’-	-‘’-	-‘’-	I and IV	EU908037 and KM374784
FIN05/E13	-‘’-	Finland, Ostrobothnia	-‘’-	ND	I	EU908038
FIN05/E14	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	EU908039
FIN05/E15	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	EU908040
FIN05/F16	-‘’-	-‘’-	-‘’-	High	-‘’-	EU908041
FIN05/F17	-‘’-	-‘’-	-‘’-	High	I and IV	EU908042 and KM374812
A1	-‘’-	Sweden, Blekinge	1995	ND	(Olofsson et al., 1999)	AF107626
A2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107627
B1	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107628
B2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107629
B3	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107630
C1	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107631
D1	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107632
D2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107633
E1	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107634
E2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107635
F1	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107636
F2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107637
F3	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107638
G1	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107639
H1	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107640
H2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107641

Strain	Species	Country, region	Isolation year	Pathogenicity	Reference	GenBank acc. no.
H3	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107642
I1	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107643
I2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107644
J1	-‘’-	Sweden, Halland	-‘’-	-‘’-	-‘’-	AF107645
K1	-‘’-	Sweden, Skaraborg	-‘’-	-‘’-	-‘’-	AF107646
K2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107647
L1	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107648
L2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107649
M1	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107650
M2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107651
N1	-‘’-	Sweden, Skåne	1997	-‘’-	-‘’-	AF107652
N2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107653
N3	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107654
O1	-‘’-	Sweden, Älvsborg	-‘’-	-‘’-	-‘’-	AF107655
O2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107656
P1	-‘’-	Finland, Ostrobothnia	-‘’-	-‘’-	-‘’-	AF107657
Q1	-‘’-	-‘’-	1996	-‘’-	-‘’-	AF107658
R1	-‘’-	-‘’-	1997	-‘’-	-‘’-	AF107659
R2	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	AF107660
Nevi18_NO06[c]	Feral American mink	Finland, North Ostrobothnia	2006	ND	IV	KM374772
Nevi23_NO07	-‘’-	-‘’-	2007	-‘’-	-‘’-	KM374790
Nevi45_NO08	-‘’-	-‘’-	2008	-‘’-	-‘’-	KM374813
Nevi47_SK08	-‘’-	Finland, South Karelia	-‘’-	-‘’-	-‘’-	KM374794
Mupu87_EE08	Polecat	Estonia	-‘’-	-‘’-	IV and (Leimann et al., 2015)	KM374800
Mama106_EE08	Pine marten	-‘’-	-‘’-	-‘’-	-‘’-	KM374780
Meme125_EE07	Badger	-‘’-	2007	-‘’-	-‘’-	KM374801
Meme132_EE08	-‘’-	-‘’-	2008	-‘’-	-‘’-	KM374803
Meme133_EE08	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	KM374804
Meme134_EE08	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	KM374805
Mama147_EE08	Pine marten	-‘’-	-‘’-	-‘’-	-‘’-	KM374807
Mama161_EE07	-‘’-	-‘’-	2007	-‘’-	-‘’-	KM374806
Nevi229_EE09	Feral American mink	-‘’-	2009	-‘’-	-‘’-	KM374808
Nevi232_EE08	-‘’-	-‘’-	2008	-‘’-	-‘’-	KM374809
Nevi238_PS09	-‘’-	Finland,	2009	-‘’-	IV	KM374776

Strain	Species	Country, region	Isolation year	Pathogenicity	Reference	GenBank acc. no.
		Pohjois-Savo				
Nevi249_ES08	-‘’-	Finland, Etelä-Savo	2008	-‘’-	-‘’-	KM374810
Nevi251_KH08	-‘’-	Finland, Kanta-Häme	-‘’-	-‘’-	-‘’-	KM374797
Nevi266_KH09	-‘’-	-‘’-	2009	-‘’-	-‘’-	KM374811
Nevi267_NK09	-‘’-	Finland, North Karelia	-‘’-	-‘’-	-‘’-	KM374792
Nevi275_SK09	-‘’-	Finland, South Karelia	-‘’-	-‘’-	-‘’-	KM374793
Nevi326_KH10	-‘’-	Finland, Kanta-Häme	2010	-‘’-	-‘’-	KM374774
Nevi358_CF08	-‘’-	Finland, Central Finland	2008	-‘’-	-‘’-	KM374778
Meme360_CF08	Badger	-‘’-	-‘’-	-‘’-	-‘’-	KM374802
Mupu363_CF08	Polecat	-‘’-	-‘’-	-‘’-	-‘’-	KM374791
Nevi435_SO13	Feral American mink	Finland, South Ostrobothnia	2013	-‘’-	-‘’-	KM374773
Meme442_SO13	Badger	-‘’-	-‘’-	-‘’-	-‘’-	KM374796
Nevi454_SK13	Feral American mink	Finland, South Karelia	-‘’-	-‘’-	-‘’-	KM374795
Nevi456_U13	-‘’-	Finland, Uusimaa	-‘’-	-‘’-	-‘’-	KM374798
Nevi458_U13	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	KM374799
Nevi479_SO13	-‘’-	Finland, South Ostrobothnia	-‘’-	-‘’-	-‘’-	KM374781
FIN12/G18	-‘’-	Finland, Ostrobothnia	2012	ND	-‘’-	KM374777
FIN13/H19	-‘’-	-‘’-	2013	-‘’-	-‘’-	KM374786
FIN13/I20	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	KM374788
FIN13/J21	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	KM374789
FIN13/K22	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	KM374779
FIN13/K23	-‘’-	-‘’-	-‘’-	-‘’-	-‘’-	KM374775
FIN14/L24	-‘’-	-‘’-	2014	-‘’-	-‘’-	KM374787
AMDV-LN1	-‘’-	China	2009	High	(Li et al., 2012)	GU108231
AMDV-LN2	-‘’-	China	-‘’-	High	-‘’-	GU108232
AMDV-G	-‘’-	USA	Late 1970s	None	(Bloom et al., 1980; Bloom et al., 1990; Huang et al., 2012)	M20036 and JN040434
AMDV-Utah1	-‘’-	USA, Utah	1963	High	(Bloom et al., 1988; Gottschalck et al., 1994)	X77083 and Z18276

Strain	Species	Country, region	Isolation year	Pathogenicity	Reference	GenBank acc. no.
AMDV-SL3	-‘’-	Germany	Early 1980s	Moderate	(Haas et al., 1990; Schuierer et al., 1997)	X97629
AMDV-K	-‘’-	Denmark	1982	High	(Gottschalck et al., 1994)	X77084
AMDV-United	-‘’-	USA	ND	High	(Gottschalck et al., 1994)	X77085

[a] Abbreviation of the country, succeeded by farm identification and animal number.
[b] No data.
[c] Abbreviation of the Latin name, succeeded by number identification, abbreviation of the region or country, and year of isolation. Nevi, *Neovison vison,* American mink; Mupu, *Mustela putorius,* polecat; Mama, *Martes martes,* pine marten; Meme, *Meles meles,* badger.

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