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Research Article

Multi-host dispersal of known and novel carnivore amdoparvoviruses

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Abstract

Amdoparvoviruses (family *Parvoviridae*) are ssDNA viruses that cause an immune complex-mediated wasting syndrome in carnivores. They are multi-host pathogens and cross-species infection is facilitated by the fact that viral entry is mediated by cellular Fc receptors recognizing antibody-coated viruses. We developed a pan-amdoparvovirus PCR and screened tissue samples from 666 wild carnivores (families Felidae, Canidae, and Mustelidae) from Newfoundland or Labrador (Canada) and molecularly characterized the identified strains. Fifty-four out of 666 (8.1%) animals were amdoparvovirus-positive. Infection rate was the highest in American mink (34/47, 72.3%), followed by foxes (Arctic and red foxes, 13/311, 4.2%), lynx (2/58, 3.5%), and American martens (5/156, 3.4%). No virus was detected in samples from 87 coyotes and 17 ermines. Viruses from Newfoundland were classified as Aleutian mink disease virus (AMDV). Mink harvested near AMDV-affected fur farms had higher prevalence (24/24, 100%) than other mink (10/23, 43.5%; $P < 0.001$) and their viruses were phylogenetically closely related to those from farms, while most viruses from other mink were in other clades. Strains from three foxes and two lynx were highly related to mink strains. This proves that farms disperse AMDV that subsequently spreads among wild mink (maintenance host) and transmits to other spillover carnivore hosts. In Labrador two novel viruses were identified, Labrador amdoparvovirus 1 (LaAV-1) found in foxes (9/261, 3.5%) and martens (5/156, 3.4%), and LaAV-2 found in one fox (0.4%). LaAV-1 fulfills all requirements to be classified as a novel species. LaAV-1 was most similar to viruses of mink and skunks (AMDV and skunk amdoparvovirus (SKAV)) while LaAV-2 was more closely related to other viruses infecting canids. LaAV-1 capsid proteins were almost indistinguishable from those of AMDV in some regions, suggesting that LaAV-1 could be a virus of mustelids that can infect foxes. While intensive farming practices provide occasions for inter-species transmission in farms, niche overlap or predation could explain cross-species transmission in the wild, but competition among

sympatric species reduces the chances of direct contacts, making this an infrequent event. Pan-amdoparvovirus detection methods in wide epidemiological investigations can play a crucial role in defining amdoparvoviral ecology and evolution and discovering novel viruses.

Key words: amdoparvovirus; Aleutian mink disease virus; parvovirus; cross-species transmission; novel virus; wildlife.

1. Introduction

Amdoparvovirus is one of the ten currently established genera within the sub-family *Parvovirinae* (family *Parvoviridae*), a group of small, non-enveloped ssDNA viruses capable of infecting vertebrates (Cotmore et al. 2019; Pénzes et al. 2020). Amdoparvoviruses possess a simple, monosense genome that includes only two main coding elements under the regulation of a single promoter. Alternative splicing of a single pre-messenger RNA generates five open reading frames (ORFs) with those formed from the left side of the genome encoding the non-structural proteins NS1, NS2, and NS3 and those from the right side coding for the capsid proteins VP1 and VP2. The three NS proteins share the same N-terminal amino acids but differ on the C-terminal side, while the two capsid proteins share the same sequence but VP2 is approximately 40 amino acids shorter on the N-terminal side. These coding elements are flanked by two short terminal untranslated regions that are characterized by the presence of palindromic sequences that fold into hairpin structures; because both these terminal sequences and the structures they form are unrelated to one another amdoparvoviruses are defined as heterotelomeric (Canuti, Whitney, and Lang 2015; Cotmore et al. 2019).

These viruses infect carnivores and cause a sometimes-fatal immune complex-mediated progressive wasting syndrome associated with hypergammaglobulinemia known as Aleutian disease. However, respiratory disorders have also been reported, especially in seronegative mink kits in which the virus causes interstitial pneumonia, and asymptomatic infections seem to be common (Alexandersen et al. 1987; Park, Best, and Bloom 2005; Jensen et al. 2012; Canuti, Whitney, and Lang 2015; Britton et al. 2017; Alex et al. 2018). In mink it has been demonstrated that disease severity depends on the infecting strain and is also influenced by host factors. In fact, clinical symptoms can be prevented by immunosuppressive treatment and the clinical outcome varies in mink with different coat colors (Bloom et al. 1975; Hadlow, Race, and Kennedy 1983; Canuti, Whitney, and Lang 2015). Since one of the target cells of viral replication are macrophages and viral entry is probably mediated by cellular Fc receptors recognizing antibody-coated viral particles, antibodies against the virus enhance viral replication, a phenomenon called antibody-dependent enhancement (ADE) (Kanno, Wolfinbarger, and Bloom 1993; Dworak, Wolfinbarger, and Bloom 1997). This entry mechanism, which does not require the interaction with a specific virus-recognized receptor, is also likely linked to the broad host-range that characterizes these viruses (Canuti et al. 2020). However, the fact that some viral strains can replicate *in vitro* and the proven capability of amdoparvoviruses to infect alveolar type II cells of the lungs suggest that specific cellular receptors may also allow viral entry (Park, Best, and Bloom 2005; Canuti, Whitney, and Lang 2015). Similarities between receptors of carnivores may also be linked to the capability of amdoparvoviruses to infect various hosts, as reported for other parvoviruses (Hueffer et al. 2003).

Although endogenous amdoparvoviral elements have been found in rodents (Pénzes et al. 2018) and an amdoparvoviral genomic fragment was recently identified in swabs from one Chinese rat (Wu et al. 2018), all five amdoparvoviral species fully characterized so far have members of the mammalian order Carnivora as their hosts (Pénzes et al. 2020). The Aleutian mink disease virus (AMDV), species *Carnivore amdoparvovirus* 1, was the sole member of this genus (formerly known as *Amdovirus*) until the early 2010s (Canuti, Whitney, and Lang 2015). AMDV was the first discovered amdoparvovirus and the disease it causes in mink has been known to farmers since the 1950s (Hartsough and Gorham 1956; Gorham, Leader, and Henson 1964). This virus has been a major concern for mink farmers ever since because of the high mortality and reproduction reduction associated with infection in farmed mink and because of the consequent economic losses (Canuti et al. 2016; Ryt-Hansen et al. 2017a). Although mink seem to be the primary hosts for this virus, other animal species represent spillover hosts (Canuti et al. 2020) and, in recent years, several studies on wild animals have identified a broad range of additional hosts (Welchman Dde, Oxenham, and Done 1993; Mañas et al. 2001; Fournier-Chambrillon et al. 2004; Farid 2013; Canuti, Whitney, and Lang 2015; Knuuttila et al. 2015; Canuti et al. 2020).

Other amdoparvoviruses whose presumed primary hosts are close relatives to mustelids (superfamily: Musteloidea) are the skunk amdoparvovirus (SKAV) and the red panda amdoparvovirus (RpAPV), classified respectively as *Carnivore amdoparvovirus* 4 and 5. So far, SKAV has been identified in wild skunks and mink (Britton et al. 2017; Canuti et al. 2017a), while the presence of RpAPV has only been demonstrated in captive red pandas (Alex et al. 2018).

The currently available information indicates that both AMDV and SKAV are widespread in North America (Nituch et al. 2011, 2012; Farid 2013; Canuti, Whitney, and Lang 2015; Nituch et al. 2015; Canuti et al. 2017a; Giannitti et al. 2018; Glueckert et al. 2019) and that AMDV is also widely diffused in European wildlife (Mañas et al. 2001; Knuuttila et al. 2015; Leimann et al. 2015; Persson et al. 2015; Jakubczak et al. 2017). However, it is most likely that AMDV was inadvertently introduced in Europe as consequence of animal trading (Canuti, Whitney, and Lang 2015). In fact, American mink were imported from North America for fur farming and accidental escape or deliberate release of animals caused this species to become endemic in many European countries (Macdonald and Harrington 2003; Jensen et al. 2012; Canuti, Whitney, and Lang 2015; Kashtanov and Salnikova 2018).

Finally, three different amdoparvoviruses have been identified in the Canidae. The gray fox amdovirus, species *Carnivore amdoparvovirus* 2, was identified in two gray foxes at a wildlife rehabilitation center in California (Li et al. 2011), the raccoon dog and fox amdoparvovirus (RFAV), species *Carnivore amdoparvovirus* 3, was detected in sick Chinese farmed raccoon dogs and Arctic foxes (Xi-Qun Shao et al. 2014), and the red fox fecal amdovirus (RFFAV), which does not have a specific species designation as it has only been partially sequenced, was found in a dead wild red fox in the Basque Country in Spain (Bodewes et al. 2014). These viruses may have a wider host range and some of

them have only been studied in captive animals, raising questions about their possible host distributions among wildlife.

Given the diversity of amdoparvoviral species that are possibly capable of cross-species transmission, it is of crucial importance to investigate the distribution of these viruses in different hosts to acquire a better understanding of their ecology and evolution. In this study, we developed a pan-amdoparvovirus screening protocol and applied it to wild carnivores within three different Carnivora families — Felidae, Canidae, and Mustelidae — to evaluate viral dispersion in wildlife and investigate amdoparvoviral potential for cross-species transmission.

2. Material and methods

2.1 Sample collections

All samples for this investigation were collected in the easternmost province of Canada, Newfoundland and Labrador (NL), the largest of the Atlantic Canadian provinces. NL is composed of an island portion located in the Gulf of St. Lawrence, Newfoundland, and a continental region attached to the eastern part of the Canadian mainland (Labrador). The two regions are about 18 km apart at the closest point and the two systems are for the most part isolated, with animals occasionally crossing ice bridges or moving from the mainland to Newfoundland on icebergs or ice floes. Among its rich wildlife, NL counts numerous autochthonous and introduced Carnivora species (Bangs 1898; South 1983, www.flr.gov.nl.ca).

This study included samples collected from a total of 666 carnivores from the families Canidae, Felidae, and Mustelidae. All American mink (*Neovison vison*) currently living in the wild in Newfoundland are either recently escaped from farms or descendant from past escapees. We were not able to distinguish between these two categories of wild-living mink, so all these animals will be referred to as wild. Overall, samples from 407 animals from Labrador and 259 animals from Newfoundland were available and in total we tested 311 foxes (red fox: *Vulpes vulpes*; Arctic fox: *Vulpes lagopus*), eighty-seven coyotes (*Canis latrans*), fifty-eight lynx (*Lynx canadensis subsolanus*), 146 martens (*Martes americana*), seventeen ermines (*Mustela erminea*), and forty-seven American mink. Sample details are given in Table 1. Samples were obtained either from licensed trappers or wildlife regional offices and animals were either killed for commercial purposes or found dead. Scientific research permits were provided by the Government of Newfoundland and Labrador and the Nunatsiavut Government where required (WLR2018-39, WLR2018-40, WLR2018-43, WLR2018-45). This study was carried out in accordance with guidelines of the Canadian Council on Animal Care, with approved protocols (14-04-AL and 15-04-AL) from the Memorial University Institutional Animal Care Committee.

2.2 Screening through a pan-amdoparvovirus hemi-nested PCR

Entire spleens, lymph nodes, or pieces of muscles (hind quarter skeletal muscle) were harvested by trappers or animal technicians at local wildlife centers, wrapped individually in Whirl-pak sampling bags and shipped frozen to Memorial University for further processing. A small fragment of tissue was then removed with disposable razor blades and used immediately for DNA isolations. DNA was isolated from approximately 10 mg spleen or 25 mg lymph node and muscle tissues with the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's specification and 5 µl (for archived DNA isolated from muscle) or 2.5 µl DNA solution was used as input for the hemi-nested PCR. Primers were designed after aligning all available amdoparvoviral sequences to match within a highly conserved region of the VP2 gene to allow for the detection of all known amdoparvoviruses and potentially novel ones (Supplementary Fig.S1). The first PCR amplified an 814-nt DNA fragment with primers P1F (5′-CCAACAAGTAATGACACCWTGG-3′) and P2R (equimolar mixture of 5′-CTCCAGYAAAGTAA CTACC-3′ and 5′-GTCCACCAACAAAGTAACTACC-3′), while the second PCR was performed using 2.5 µl product of the first amplification round and amplified a 315-nt fragment with primers P1F and P1R (5′-GTTGGTTTRGTTGCTCTCC-3′). PCR mixtures included 12.5 µl of DreamTaq Green PCR Master Mix (ThermoFisher Scientific), 0.5 µM of each primer and water to a final volume of 25 µl. Amplification included an initial denaturation step at 95 °C for 3 min, followed by thirty-five (first round) or twenty-five (second round) cycles of 95 °C for 30 s, 50 °C for 30 seconds, and 72 °C for 50 (first round) or 30 (second round) seconds, and a final elongations step at 72 °C for 4 min. Since viral load varied considerably between samples, the products of the first PCRs were loaded on 1 per cent agarose gels and negative samples were used for the nested amplification. All putative positives were confirmed by sequencing.

2.3 Sequencing

To be able to compare identified viral strains to sequences previously obtained in Newfoundland, a small fragment of the NS1 gene was amplified from positive samples with primers ScF and ScR as described previously (Canuti et al. 2016). Furthermore, the complete genome was obtained for a selection of strains by performing overlapping PCRs. All primers used for complete genome sequencing are available in (Canuti et al. 2016). Amplified products were purified with AMPure Beads (Beckman Coulter) and sent for Sanger sequencing at The Center for Applied Genomics (Toronto, Canada). When sequences showed a high number of double peaks, indicative of co-infection, corresponding amplicons were cloned into pGEM®-T Easy (Promega) vector followed by sequencing of colony PCR products obtained with the M13 primers that bind adjacent to the plasmid cloning site.

2.4 Sequence analyses

Sequences obtained in this study were compared to all amdoparvoviral sequences available in the GenBank database (https://www.ncbi.nlm.nih.gov) as of 3 May 2020 (N = 1,494) and different sequence sets were used for the different analyses. The region of the poly-glycine stretch at the beginning of the VP2 protein was removed from all analyses as it is a repeat of highly variable length and causes issues during sequence alignments. Sequence alignments were obtained with Clustal W (Larkin et al. 2007) and used for phylogenetic inference using MEGA X (Kumar et al. 2018). Trees were built with the maximum-likelihood method (Felsenstein 1981) using the best model for distance estimation identified by a modeltest analysis and robustness of clades was assessed with a bootstrap test (100 and 1,000 replicates for large and small datasets, respectively). Genetic similarities (1 − p-distance) between strains and between and within clades were calculated with MEGA X and alignments were inspected for recombination using the recombination detection program (RDP) version 5 (Martin et al. 2015) as previously described (Canuti et al. 2016).

Table 1. Details of samples used in this study.

Location	Species	Sample type	No.[a]	Trapping year	Reference
Labrador	*V. vulpes*	DNA from muscle	47	2015–6	Alanazi (2020)
	V. vulpes	DNA from muscle	82	2012–4	Nadin-Davis et al. (submitted)
	Fox (species unknown)	DNA from muscle	3	2012–4	Nadin-Davis et al. (submitted)
	V. vulpes	Head lymph node	27	2015–7	This study
	V. lagopus	Spleen	1	2018	Bouchard et al. (in preparation)
	V. vulpes	Spleen	62	2018	Bouchard et al. (in preparation)
	Fox (species unknown)	Spleen	29	2018	Bouchard et al. (in preparation)
	V. vulpes	Spleen and head lymph node	10	2018	Bouchard et al. (in preparation)
	M. americana	Spleen	146	2016–7	This study
Newfoundland	*V. vulpes*	Spleen	15	2014	Canuti et al. (2016)
	V. vulpes	Spleen	35	2015–8	This study
	C. latrans	Spleen	85	2014	Canuti et al. (2017b)
	C. latrans	Spleen	2	2016	This study
	L. c. subsolanus	Spleen	38	2012–5	Canuti et al. (2017b) and Hendrikse et al. (2019)
	L. c. subsolanus	Spleen	20	2014–7	This study
	M. erminea	Spleen	17	Unknown	Canuti et al. (2016)
	N. vison	Spleen	10	2014	Canuti et al. (2016)
	N. vison	Spleen	37	2015–6	This study

[a]Number of individual animals tested.

2.5 Statistical analyses

Differences between viral positivity rates in different host populations (number of positive animals over the total number of individuals tested) were evaluated for statistical significance using the Mid-p exact test with OpenEpi (Dean, Sullivan, and Soe 2013) and P-values ≤ 0.05 (two-tailed tests) were considered significant.

3. Results

3.1 Viral prevalence and host distribution

In total, fifty-four out of 666 (8.1%) animals were amdoparvovirus-positive. Viral prevalence was significantly higher in Newfoundland (15.1% vs. 3.7%, $P < 0.001$), but this was likely due to the high infection rate in Newfoundland mink (72.3%). Some of the mink were collected in proximity to fur farms known to be affected by AMDV and in this group viral prevalence reached 100 per cent (24/24), while in mink harvested in other areas prevalence was significantly lower (10/23, 43.5%; $P < 0.001$). This indicates that either mink farms are a substantial source of viruses for wild animals or that a high proportion of mink that are found near mink farms are escaped animals.

Among other mustelids, five of the 146 (3.4%) martens from Labrador were positive and no virus was identified in ermines. Within the Canidae, 4.2 per cent of the foxes (thirteen out of 311) were positive and no virus was found in coyotes. Prevalence was higher in foxes from Newfoundland (6%) compared to Labrador (3.8%), although not significantly ($P = 0.5$), and all positives were red foxes. Finally, within the Felidae, amdoparvoviruses were identified in two lynx with a prevalence of 3.5 per cent. Screening results are summarized in Table 2.

Table 2. Amdoparvoviral prevalence among different hosts in NL.

Location	Host	Number tested	Number positive	Prevalence (%)
Labrador	Fox	261	10[a]	3.8
	Marten	146	5	3.4
	Total	407	15	3.7
Newfoundland	Fox	50	3	6
	Coyote	87	0	0
	Lynx	58	2	3.5
	Mink	47	34	72.3
	Ermine	17	0	0
	Total	259	39	15.1
Total		666	54	8.1

[a]All positive samples were from *V. vulpes*; three in lymph nodes and seven in muscle.

3.2 Virus classification and identification of a novel amdoparvoviral species

The full viral coding sequence was obtained for a selection of viruses from positive mink ($N = 7$), including most strains from animals trapped further away from farms and two strains from animals trapped near farms, martens ($N = 4$), and a Newfoundland fox. We also sequenced the complete VP of two viruses from Labrador foxes and the complete NS1 of an additional mink virus. Unfortunately, despite extensive efforts, due to suboptimal sample type (muscle) or low viral load we could only obtain partial sequences for the two lynx viruses and most of the fox viruses.

To classify the identified strains, the obtained complete NS1 and VP2 predicted protein sequences were aligned to all amdoparvoviral complete NS1 ($N = 326$) and VP2 ($N = 305$) sequences available in GenBank and used to build genus-wide phylogenetic trees (Supplementary Figs S2 and S3). Based on these phylogenies, a subset of sequences was selected to build the simplified trees shown in Fig. 1. All sequences from Newfoundland consistently clustered with other AMDV strains and were, therefore, all classified as *Carnivore amdoparvovirus 1*. However, the NS1 proteins of viruses from Labrador were sufficiently distinct from those of all other amdoparvoviruses to form a separate and highly supported (bootstrap: 100) clade,

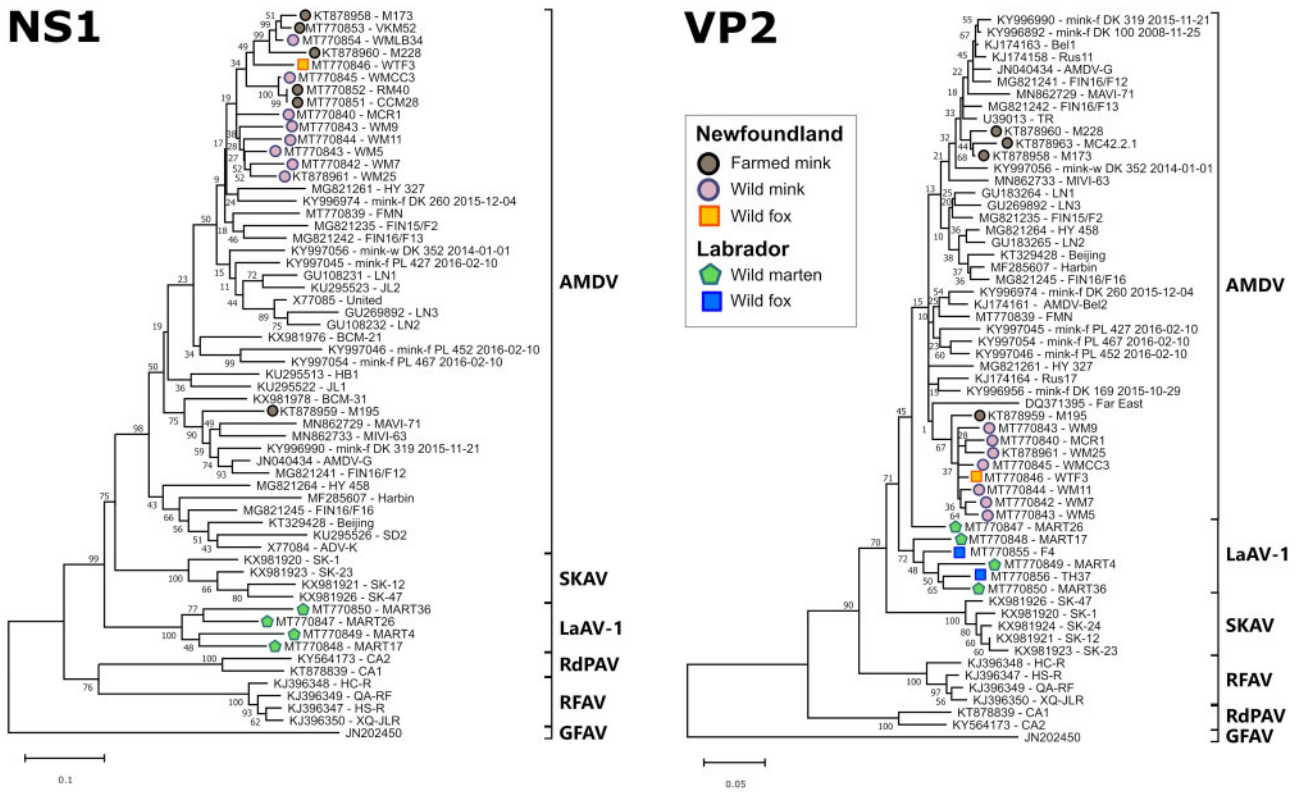

Figure 1. Phylogenetic analyses of NS1 and VP2 proteins of members of the genus *Amdoparvovirus*. Evolutionary histories were inferred with the maximum-likelihood method (Felsenstein 1981) based on the LG model (Le and Gascuel 2008) in MEGA X (Kumar et al. 2018). A discrete Gamma distribution was used to model evolutionary rate differences among sites (+G, parameter = 0.5839 and 0.4481 for NS1 and VP2, respectively) and the rate variation model allowed for some sites to be evolutionarily invariable (+I, 17.03% and 30.90% of sites for NS1 and VP2, respectively). The outcome of the bootstrap analysis (1,000 replicates) (Felsenstein 1985) is shown next to the nodes and branch lengths are proportional to genetic distances as indicated by the scale bar. Strains identified in NL are labeled with shapes and colors as indicated in the legend and viral classifications, based on NS1 sequence analyses, are indicated on the right of each tree.

indicating they represent a previously undetected virus, which we named Labrador amdoparvovirus 1 (LaAV-1). However, the distinction between AMDV and LaAV-1 strains was somewhat lost in the trees built with VP2 protein sequences, where viruses from these two groups were all included in a single, although poorly supported, clade. Nevertheless, within this clade two viruses from foxes (F4 and TH37) and two from martens (MART4 and MART36) consistently grouped together, indicating that marten and foxes from Labrador are infected by similar viruses.

To define the classification of the new virus, between- and within-clade pairwise sequence comparisons were performed for the two proteins (Supplementary Tables S1 and S2). The NS1 proteins of LaAV-1 strains were, on average, 66.6–79.3 per cent identical to those of other amdoparvoviruses, and were most closely related to those of AMDV (79.3%) and SKAV (78.3%). Although the average NS1 pairwise sequence identity within the LaAV-1 group was the lowest (86.1%), within the AMDV clade pairwise sequence identities were as low as 78.3 per cent, indicating a wider within-group variability (Supplementary Table S1). Although this could signify the existence of multiple AMDV sub-lineages, no bootstrap-supported sub-clade was consistently found across phylogenies. The results of these analyses confirmed that the distinction between AMDV and LaAV-1 in the VP2 genomic portion is not as evident as in the NS1 region, with pairwise sequence identity values being sometimes higher between rather than within the two groups (Supplementary Table S2). Overall, VP2 proteins were much more conserved than NS1 proteins, both within and between species. Finally, when we screened an alignment of 304 complete genomes for recombination with RDP, we did not find any evidence for recombination events involving LaAV-1 strains.

The fully sequenced genome of LaAV-1 shows all elements that characterize amdoparvoviruses, including conserved ORFs, the typical splicing profile, and the presence of the distinctive helicase superfamily 3 (SF3) domain within NS1. Furthermore, since the ICTV criteria for parvovirus classification are based on NS1 protein phylogeny and sequence identities with the demarcation limit for species set at 85 per cent (Pénzes et al. 2020), LaAV-1 should be considered a novel amdoparvoviral species.

3.3 AMDV ecology and inter-host transmission

All AMDV-positive samples were from animals from Newfoundland and these included thirty-four mink, three foxes, and two lynx (Supplementary Table S3). All mink were from an area of southeastern Newfoundland known as the Avalon Peninsula, and this is also where the three positive foxes and one of the two positive lynx were trapped. The other positive lynx was from central Newfoundland while none of the animals that were from other locations (western or northern Newfoundland) was amdoparvovirus-positive (Supplementary Table S4). However, the only samples available from mink were from the Avalon Peninsula, where about 50 per cent of the total number of samples with a known origin was collected. Nevertheless, this may indicate that amdoparvoviruses are not spread throughout the entire island and demonstrates that the same viruses infect different hosts.

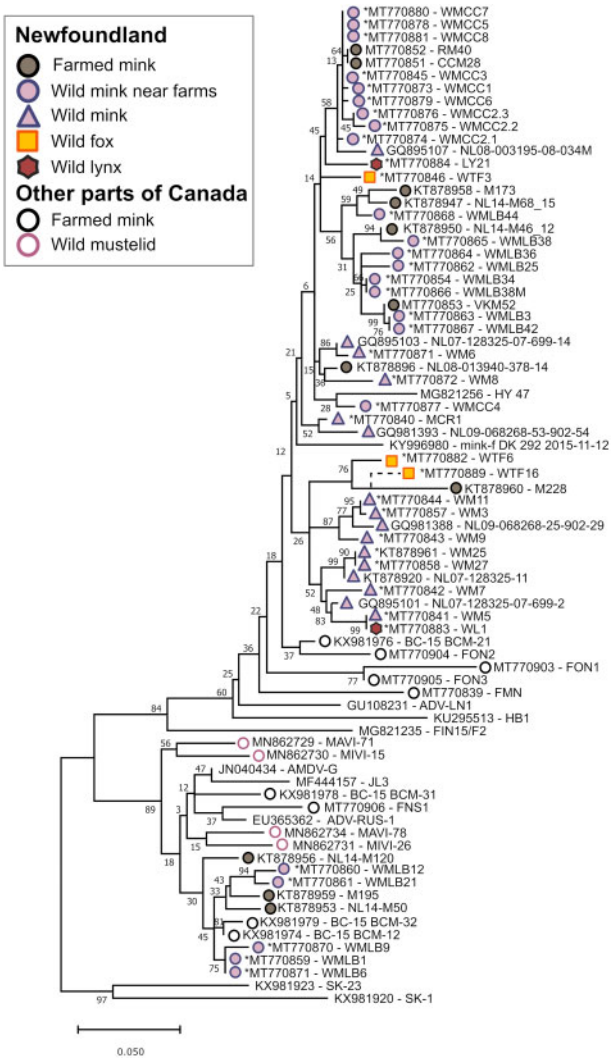

Figure 2. Molecular epidemiology of amdoparvoviruses from Newfoundland. The phylogenetic tree is based on a 423-nt fragment of the NS1 gene (positions 1253-1672 of the reference strain AMDV-G, accession number: JN040434) and was obtained with the maximum-likelihood method (Felsenstein 1981), based on the Kimura 2 parameters model (Kimura 1980) in MEGA X (Kumar et al. 2018). A discrete Gamma distribution was used to model evolutionary rate differences among sites (+G, parameter = 0.6046) and the rate variation model allowed for some sites to be evolutionarily invariable (+I, 40.06% of sites). The outcome of the bootstrap analysis (1,000 replicates) (Felsenstein 1985) is shown next to the nodes and branch lengths are proportional to genetic distances as indicated by the scale bar. Strains identified in Newfoundland and other parts of Canada are labeled with shapes and colors as indicated in the legend and viruses from this study are marked with asterisks. Three sequences were obtained from sample WMCC2 after molecular cloning and these are indicated with a dot followed by the clone number. The phylogenetic placement of the partial VP2 of strain WTF16 relative to other AMDVs based on a different analysis (Supplementary Fig. S4) is shown with a dotted line. SKAV strains (SK-1 and SK-23) were used as an outgroup.

To study the molecular epidemiology of these viruses in Newfoundland, a phylogenetic tree was built that included fifty-four partial NS1 sequences of strains from this and a previous study (Canuti et al. 2016) obtained from wild and farmed Newfoundland animals (Fig. 2). Since it was not possible to obtain the NS1 sequence for one of the fox viruses (WTF16), the phylogenetic relationships of this virus with other AMDVs were evaluated with a separate phylogenetic analysis performed with a fragment of the VP2 gene (Supplementary Fig. S4) and we summarized these results by indicating the placement of this strain in the VP2 tree with a dotted line in Fig. 2. However, since the evolutionary histories of the two genetic regions are different and these viruses are known for their tendency to recombine, this might not reflect the actual placement of this strain on the NS1 tree. With one exception (WMCC4), all sequences from wild mink that were from areas neighboring affected mink farms were included in three clades that also included sequences obtained recently from local farms, although support was low (bootstrap: 45–58). Other viruses from wild mink were not as closely related to farm strains and were not included in any of these three clades. Remarkably, the strains obtained from canids and felids were very close to mink strains, both those from farmed and wild animals, providing strong evidence for cross-species transmission.

3.4 LaAV ecology and inter-host transmission

Overall, ten foxes and five martens from Labrador were amdoparvovirus-positive (Supplementary Table S5). Interestingly, viral distribution was different in the two hosts as all the positive martens were sampled in western Labrador, while the positive foxes originated from the central region (N = 6) or from eastern Labrador (N = 4). Interestingly, positivity rates in these regions were very similar (6.9–8.5%). However, while samples from foxes were evenly distributed across the whole region, approximately 66 per cent of samples from martens with known location were from western Labrador. No positive animals were found in the northern part of the region (Supplementary Table S6).

To study the molecular epidemiology of the novel viruses in this region, a phylogenetic tree was built with partial VP2 nucleotide sequences of sixteen strains identified in Labrador (Fig. 3). To retain as much sequence information as possible the tree was built with a 763-nt region of the VP2 gene, while the placement of three additional sequences from Labrador (SFP44.1, SFP49, MART122) and the virus previously identified in European red foxes (S40) was estimated based on additional analyses performed with smaller and only partially overlapping genetic regions (Supplementary Fig. S5). For illustrative purposes, the placement of these sequences is reported in Fig. 3 and is indicated by dotted lines. Despite differences in viral geographic distribution between the two hosts, martens and foxes were infected with similar viruses. In fact, all but two sequences were included within one clade (although it was poorly supported) and within this clade there was no clear sub-grouping according to host, something that is also visible in the tree based on the complete VP2 amino acid sequence of a smaller number of viruses (Fig. 1). One of the two outliers was one of the viruses from martens (MART17) that was, however, still closely related to the other viruses from Labrador. Although the phylogenetic placement of the Labrador viruses was inconsistent across phylogenies, all trees support the observation that the distinction between AMDV and LaAV-1 is less evident in VP2. However, for one of the fox viruses (F4), we could obtain a partial NS1 protein sequence and this clearly belonged to the LaAV-1 clade (Supplementary Fig. S6). The partial NS1 sequence identity of F4 with other LaAV-1 strains was 87.8–91 per cent, indicating that the viruses from foxes and martens indeed belong to the same species.

The other outlier, fox strain F6, was localized in a totally different area of the tree, clustering with two other previously identified fox viruses (GFAV and RFFAV) and the virus identified

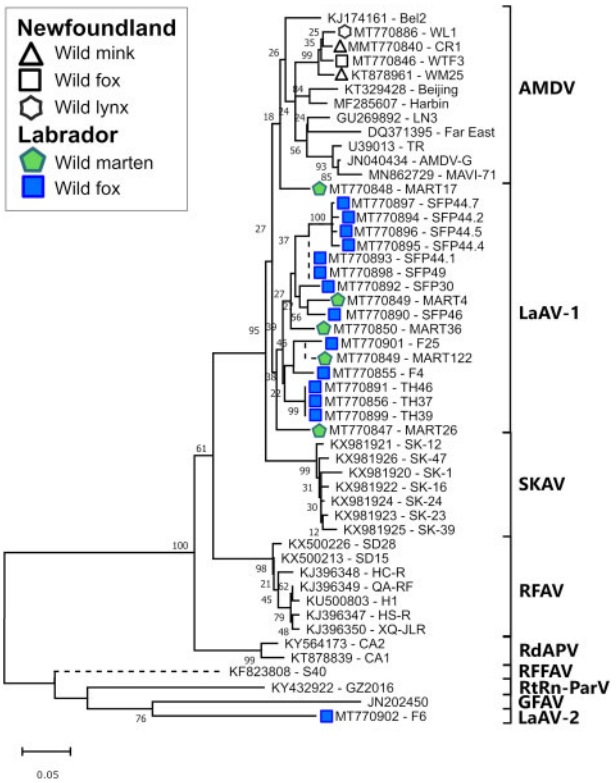

Figure 3. Molecular epidemiology of amdoparvoviruses from Labrador. The phylogenetic tree is based on a fragment of approximately 750 nt of the VP2 gene (positions 2,742–3,491 of the reference strain AMDV-G, accession number: JN040434) and was obtained with the maximum-likelihood method (Felsenstein 1981), based on the HKY model (Hasegawa, Kishino, and Yano 1985) in MEGA X (Kumar et al. 2018). A discrete Gamma distribution was used to model evolutionary rate differences among sites (+G, parameter = 0.4544). The outcome of the bootstrap analysis (1,000 replicates) (Felsenstein 1985) is shown next to the nodes and branch lengths are proportional to genetic distances as indicated by the scale bar. Five sequences were obtained from sample SFP44 after molecular cloning and these are indicated with a dot followed by the clone number. Strains identified in NL are labeled with shapes and colors as indicated in the legend. The phylogenetic placement of a shorter VP2 sequence of strains SFP44.1, SFP49, MART122, and S40 based on different analyses (Supplementary Fig. S5) is shown with a dotted line. Viral classifications are indicated on the right.

in a Chinese rat (RtRn-ParV) (Wu et al. 2018). This virus was detected in a lymph node collected from the head of a fox from eastern Labrador and this was the only sample positive for this virus. Unfortunately, we could not obtain its complete genomic sequence or the NS1 sequence that is required to provide a proper classification. However, within the short region available this virus was equidistant to the other two viruses in this clade as it was 82 per cent identical to GFAV and 81.4 per cent identical to RtRn-ParV. GFAV and RtRn-ParV were 79.7 per cent identical to each other. The distance between these viruses was greater than the distance between the same region for SKAV and AMDV, two distinct but highly related species (91.3%). We can, therefore, conclude that the F6 strain represents a second yet-uncharacterized amdoparvoviral species, which we named Labrador amdoparvovirus 2 (LaAV-2).

Finally, to search for additional similar potentially unrecognized LaAV sequences from other hosts, we aligned all sequences downloaded from the GenBank database to the LaAV strains and performed several phylogenetic analyses with partial genomic sequences. No sequence could be classified as LaAV-1 or LaAV-2, but these analyses showed that four viruses from mink labeled in GenBank as AMDV (strains SD15 and SD28 shown in Fig. 3, and viruses with accession numbers KY421414 and KY421419) were included in highly supported clades with RFAV sequences and a blast analysis confirmed these sequences were actually from the species *Carnivore amdoparvovirus* 3.

4. Discussion

4.1 Pan-amdoparvovirus PCR and novel amdoparvoviral species

The pan-amdoparvovirus PCR we developed was capable of detecting all four amdoparvoviral species whose DNA is available in our laboratory (AMDV, LaAV-1, LaAV-2, and SKAV), demonstrating its validity as a tool for performing amdoparvoviral epidemiological investigations. The three primers were designed within a very conserved part of the VP2 gene that seems to be a good target for *in vitro* amplification. In fact, PCRs in this region were successful even for low-load viruses and for strains from all species for which all other PCRs remained negative. This method also proved to be valuable for amdoparvovirus discovery and could be useful in future investigations as it allowed the discovery of two novel amdoparvoviruses.

One of the two novel viruses, LaAV-1, was very close to viral species that infect mustelids and mephitids and fulfills all requirements to be classified as a novel species. The second novel virus, LaAV-2, was only partially sequenced as all our attempts to obtain further sequence information, including specific PCRs (Canuti et al. 2016, 2017a), genome walking (Canuti et al. 2019), and virus discovery methods (Verhoeven et al. 2018), were unsuccessful. This virus is highly divergent from all other known amdoparvoviruses and this explains why available primers, designed based on the genomic sequences of other species, did not produce noticeable amplification outside the most well-conserved VP2 fragment. Furthermore, PCRs using the sample in which this virus was detected would only result in a band after the second round of amplification, indicative of low viral load. Nevertheless, our preliminary data demonstrate that this virus is divergent enough from other viruses to potentially represent a separate species and future screening efforts should be directed towards identifying positive samples suitable for complete genomic sequencing.

Our sequence analyses also highlight how viral diversity within the AMDV group is higher than for other amdoparvoviral species, and in certain phylogenies multiple intermediate clades between AMDV and other viruses are observed. Indeed, NS1 pairwise amino acid sequence identity within the AMDV group was as low as 78.3 per cent, lower than the cut-off for species demarcation (85%). This may indicate the existence of several lineages, but no sub-clade that was statistically supported and consistent across phylogenies could be identified. Poor phylogenetic consistency and low bootstrap support, especially in the most conserved VP2 region, are a well-reported occurrence when dealing with partial sequences of AMDVs and it is in part a consequence of the highly recombinant nature of these viruses (Canuti et al. 2016, 2017a; Virtanen et al. 2019). Indeed, when we analyzed an alignment of 304 full amdoparvovirus genomes, RDP identified over forty potential recombination events, some of which were already reported (Canuti et al. 2016, 2017a; Virtanen et al. 2019), although none of these involved the newly identified viruses. Since chimeric genomes and a yet unknown viral diversity remain obstacles in accurately determining evolutionary relationships among AMDV-like viruses,

intensifying sequencing efforts would greatly facilitate resolving the phylogeny of amdoparvovirus.

4.2 Amdoparvoviruses as multi-host pathogens

Based on our results and those from previous studies (Mañas et al. 2001; Farid 2013; Xi-Qun Shao et al. 2014; Knuuttila et al. 2015; Nituch et al. 2015; Canuti et al. 2017a, 2020; Virtanen et al. 2019) we can affirm with reasonable certainty that amdoparvoviruses are multi-host viruses and this may be linked, at least in part, to the antibody-mediated cell entry mechanism. A predominance of negative selection pressure acting on the VP2 protein and a surprisingly higher degree of diversifying selection pressure acting on non-structural compared to structural proteins was previously demonstrated for AMDV (Canuti et al. 2016). As the host immune response contributes to the infection process by allowing viruses coated with antibodies to enter target cells and favoring viral replication, we believe that this stability of the proteins that are target of the immune response may be beneficial for the virus. This could also explain why, contrary to most other parvoviruses (Pénzes et al. 2020), pairwise identities are higher for VP2 than NS1, both within and between amdoparvoviral species, suggesting that all amdoparvoviruses may use this mechanism to enter cells and that ADE may be important for all of these viruses.

AMDV and AMDV-like viruses have been found in a wide range of hosts within the carnivore families Mustelidae, Felidae, Procyonidae, and Mephitidae (Mañas et al. 2001; Farid 2013; Canuti, Whitney, and Lang 2015; Knuuttila et al. 2015; Nituch et al. 2015; Canuti et al. 2020). To the best of our knowledge, this study is the first to detect AMDV in red foxes, proving that AMDV is capable of infecting canids, and in lynx. Furthermore, our results strengthen the previous hypothesis (Canuti et al. 2020) that mink are the primary hosts of AMDV, enable its persistence, and are the source of viruses for other susceptible species, where genetically related AMDVs circulate at a lower frequency. In fact, in the studied area AMDV prevalence was significantly higher in mink (72.3%) than in both fox (6%) and lynx (3.5%). Whether spillover hosts can transmit viruses remains to be determined.

Among other amdoparvoviruses, the primary hosts for SKAV are skunks (family Mephitidae) but this virus can also infect animals in the Mustelidae (Nituch et al. 2015; Canuti et al. 2017a; Giannitti et al. 2018; Glueckert et al. 2019), while RFAV has been found in Canidae (farmed Arctic foxes and raccoon dogs) (Xi-Qun Shao et al. 2014) and our sequence analyses demonstrate it can also infect mink. However, RFAV has been identified primarily in fur farms and at this point it is impossible to determine which is its primary host and epidemiological studies focused on wildlife are necessary to individuate maintenance and spill-over hosts and elucidate cross-species transmission dynamics. Finally, we detected LaAV-1 in both canids (foxes) and mustelids (martens) and we did not observe a clear distinction between strains detected in the two hosts. Since viral prevalence was very similar in martens and foxes, we cannot make definitive conclusions about the LaAV-1 primary host, but we can make some hypotheses. Both genomic regions of this virus are more related to viruses of mustelids (AMDV) and mephitids (SKAV) than to viruses of canines (RFAV, RFFAV, GFAV, LaAV-2). Furthermore, the VP2 protein sequences of LaAV-1 are so similar to those of AMDV that they become virtually indistinguishable from them in some regions. This may indicate that LaAV-1 is a virus of mustelids that was transmitted to foxes at least once.

4.3 Intensive farming facilitates cross-species transmission and viral dispersal

Having the potential to infect different hosts is not sufficient for a virus to cross the species barrier as an uninfected animal has to have close contacts with an infected individual or with contaminated material to acquire the infection. It is easy to appreciate how viruses can be transmitted between different susceptible hosts in an intensive farm setting. There, high population densities and frequent animal turnover favor viral transmission and viruses can easily spread within and between barns through the transfer of infected animals and contaminated personnel or equipment (Espregueira Themudo et al. 2012; Prieto et al. 2017; Ryt-Hansen et al. 2017b; Cao et al. 2018). This is further facilitated by the high environmental resistance of amdoparvoviruses (Hussain, Price, and Farid 2014; Canuti, Whitney, and Lang 2015). Furthermore, in these settings a shorter generation time rapidly increases the genetic diversity of amdoparvoviruses (Mennerat et al. 2010), which are already characterized by evolutionary rates approaching those of RNA viruses (Moya, Holmes, and González-Candelas 2004; Shackelton et al. 2005; Duffy, Shackelton, and Holmes 2008) and they frequently undergo recombination to further increase this diversity (Canuti et al. 2016, 2017a; Virtanen et al. 2019), amplifying the evolutionary potential for cross-species transmission and efficient replication in a new host (Woolhouse, Taylor, and Haydon 2001).

What clearly emerges from this study is that mink farms are an important source of viruses for wild animals, as previously hypothesized (Nituch et al. 2011, 2012; Knuuttila et al. 2015; Canuti et al. 2016; Virtanen et al. 2019). There were no mink on the island of Newfoundland before they were imported for fur farming in the 1930s (Northcott, Payne, and Mercer 1974), and presumably also no AMDV. This is demonstrated by the fact that AMDV strains found in farmed and wild animals are similar to each other and also to strains found worldwide in other mink farms. Since viral prevalence in mink harvested close to a mink farm (100%) was significantly higher than the prevalence in mink from other areas (43.5%) we can conclude that virus escape from farms is still happening. This corroborates previous findings where a higher AMDV seroprevalence was observed in areas closer to mink farms than in areas farther from farms (Nituch et al. 2011). Nonetheless, since viruses found in most wild animals harvested further from farms were less identical to current strains from farms and formed separate clades, we can also conclude that AMDV circulates in wild animals at high rates independently from farms. These wild-specific strains are presumably also of farm-origin but appear to no longer circulate in farms.

4.4 Predation could explain cross-species transmission in the wild

In the wild things are more complicated as chances for cross-species transmission are limited by the reduced number of contacts between different animal species. Ecological overlap between different hosts explains how viral exchange is possible (Canuti et al. 2020). For example, niche overlaps are possible for animals with similar diets, such as mink and otters that both exploit aquatic environments (Bonesi, Chanin, and Macdonald 2004), or martens and foxes that feed on rodents (Storch, Lindstrom, and Jounge 1990; Lanszki, Zalewski, and Horváth 2007). However, although carnivores frequently share similar geographic ranges, competition among sympatric species often

results in resource partitioning and niche differentiation (May et al. 2008; Prigioni et al. 2008; Pereira et al. 2012), reducing the chances of direct contacts between animals. This may explain why cross-species transmission occurs but not as frequently as we could imagine by simply looking at overlaps in host species distributions.

The fact that foxes pursue and kill martens (Lindström et al. 1995) can also explain why these two carnivore species share the same amdoparvoviruses and substantiate our hypothesis that LaAV-1 is a mustelid virus that can be transmitted to foxes, possibly through predation. Interestingly, the recently discovered amdoparvovirus that was found in rodents (Wu et al. 2018) is highly related to viruses of foxes. This triggers the intriguing hypothesis that the carnivore amdoparvoviruses so far found exclusively in foxes are actually rodent viruses that could cross the species barrier via predation. This could also explain the extremely low prevalence of LaAV-2, which was found only once, compared to LaAV-1. Indeed, in some cases, there are differential viral distributions in the different hosts as the likelihood for host-to-host transmission is not the same in both directions (Canuti et al. 2020). This also could be explained by predation, which could favor unidirectional cross-species transmission and cause differences in infection rates in the tested host species. For example, the fact that foxes occasionally feed on mink (Carlsson et al. 2010) can offer an explanation for how cross-species transmission may occur in this system and why the infection rate in this predator was much lower than in mink. Lynx might have acquired the infection in the same way since they are known to commonly kill foxes (Helldin, Liberg, and Glöersen 2006) and might kill mink too. Nevertheless, evaluating the role of predation in amdoparvoviral cross-species transmission could certainly be an interesting future research direction.

In conclusion, our data contribute to the growing body of evidence demonstrating that amdoparvoviruses are multi-host pathogens and that several yet uncharacterized amdoparvoviral species exist. Furthermore, this study highlights how the application of pan-amdoparvovirus detection methods in wide epidemiological investigations has an important role in elucidating amdoparvoviral ecology and evolution.

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Data Availability

All sequences obtained in this study were deposited in GenBank and are available under accession numbers MT770839–MT770908.

Supplementary data

Supplementary data are available at *Virus Evolution* online.

Conflict of interest: None declared.

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