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2020-05

Virtanen , J , Aaltonen , K , Vapalahti , O & Sironen , T 2020 , ' Development and validation of nucleic acid tests to diagnose Aleutian mink disease virus ' , Journal of Virological Methods , vol. 279 , 113776 . <https://doi.org/10.1016/j.jviromet.2019.113776>

<http://hdl.handle.net/10138/321263>

<https://doi.org/10.1016/j.jviromet.2019.113776>

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PII: S0166-0934(19)30361-1

DOI: <https://doi.org/10.1016/j.jviromet.2019.113776>

Reference: VIRMET 113776

To appear in: *Journal of Virological Methods*

Received Date: 9 August 2019

Revised Date: 4 November 2019

Accepted Date: 10 November 2019

Please cite this article as: Virtanen J, Aaltonen K, Vapalahti O, Sironen T, Development and validation of nucleic acid tests to diagnose Aleutian mink disease virus, *Journal of Virological Methods* (2019), doi: <https://doi.org/10.1016/j.jviromet.2019.113776>

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Development and validation of nucleic acid tests to diagnose Aleutian mink disease virus.

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Pages: 15

Figures: 2

Tables: 3

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Highlights

- Setting up a sensitive and specific probe-based AMDV-PCR
- Comparison and validation of three diagnostic AMDV-PCRs
- Screening and detection of AMDV from mink and fox samples

Abstract

Aleutian disease (AD), caused by Aleutian mink disease virus (AMDV), causes significant welfare problems to mink, and financial losses to the farmers. As there is no vaccine or treatment available, reliable diagnostics is important for disease control. Here, we set up a probe-based real-time PCR (NS1-probe-PCR) to detect all strains of AMDV. PCR was validated and compared to two other real-time PCR methods (pan-AMDV- and pan-AMDO-PCR) currently used for AMDV diagnostics in Finland. The NS1-probe-PCR had a similar detection limit of 20 copies/reaction based on plasmid dilution series, and similar or better diagnostic sensitivity, when evaluated using spleen samples from mink, and stool samples from mink and foxes. None of the three PCR tests cross-reacted with other parvoviruses. The NS1-probe-PCR also showed

a significantly higher specificity than the pan-AMDO-PCR with spleen samples and the best specificity with stool samples. Furthermore, it produced the results more rapidly than the other two PCRs making it a promising tool for both diagnostic and research purposes.

Keywords: AMDV; diagnostics; qPCR

¹ Abbreviations: AD, Aleutian disease; AMDV, Aleutian mink disease virus; CPV, canine parvovirus; FPLV, feline panleukopenia virus; MEV, mink enteritis virus; HKY, Hasegawa Kishino Yano model

1. Introduction

Aleutian disease (AD), caused by Aleutian mink disease virus (AMDV), is one of the most significant infectious diseases of mink. AD causes both significant welfare problems to the animals and financial losses to farmers. AMDV is secreted into urine, feces and saliva of infected mink (1-3) and is transmitted vertically through placenta or horizontally in direct contact with an infected animal or in indirect contact with contaminated environment (4-6). Clinical signs vary from subclinical to severe and fatal (7) depending, among other things, on mink age and genotype (4,8).

AD was first reported in Aleutian-type mink in the USA in 1956 (9) but it has since spread to all mink producing countries and also been reported in other mink genotypes (10), ferrets and several wild mustelids and carnivores (11-14). AMDV belongs to family *Parvoviridae* (15,16) and species *Carnivore amdoparvovirus 1* (17) and its 4.7 kb ssDNA genome (16,18,19) encodes two structural proteins (VP1 and VP2) and three non-structural proteins (NS1, NS2 and NS3) (15,20-22).

Despite the eradication efforts, AMDV still causes major epidemics (23). As there is no effective treatment and the attempts to develop a vaccine have been unsuccessful (24,25), reliable diagnostics is important in disease control. Diagnostics is mostly based on pathological findings and serology, using antibody detection methods such as CIEP and ELISA, as well as DNA detection by PCR (26-28). As some mink can clear the virus, but remain antibody-positive, the best way to determine viremia (from euthanized individuals) is to perform a PCR from spleen tissue (3). However, genetic diversity makes it difficult to set up a PCR that is sensitive and able to amplify all virus strains, while remaining specific. As false positive or negative results

might have severe economic consequences to the farmers, it is important to have properly validated PCRs. Currently, there is no golden standard PCR method available, which complicates validation.

The aim of this study was to set up a diagnostic PCR that is specific, amplifies all AMDV strains, and is easy to interpret, and validate and compare that and PCRs currently used in diagnostics in Finland.

2. Materials and methods

2.1 PCR protocols

A new probe-based PCR (NS1-probe-PCR) was developed to amplify the genomic region of 1586-1645 bp (according to strain AMDV-G (GenBank accession no. M20036.1)) of all AMDV strains. Primers AMDV NS1 F (GGAAARACCYTRCTRGATCYT), and AMDV NS1 R (GTTACCRCACTCTTCASHCC), and the probe (AACTTTCCATGGACTGA) were designed so that they have a maximum of two mismatches with the sequences (for comparison, we used sequences published in GenBank by the end of 2016). PCR reactions contained 4 µl of 5x HOT FIREPol Probe Universal qPCR Mix, 1.2 µl of 10 µM forward primer, 1.2 µl of 10 µM reverse primer, 0.2 µl of 10 µM probe, 2 µl of template DNA and 11.4 µl of water. The cycling conditions were: initial denaturation in 95°C for 10 min followed by 45 cycles of denaturation in 95°C for 15 s and annealing/elongation in 60°C for 60 s. Primer AMDV NS1 F was later updated (GGAAARACCYTRCTRGATCYTT) to match all the sequences published by March 2019 and the sensitivity was tested to be similar to that of the first one. The NS1-probe-PCR was compared to two SYBR Green - based real-time-PCRs currently used in diagnostics in Finland: pan-AMDV-PCR that has been modified from the PCR by Jensen et al. (29) and amplifies the region 578–951 (30) of AMDV and pan-AMDO-PCR (11) that amplifies the region 1662–2302 of AMDV and other amdoparvoviruses. Initially, only pan-AMDV-PCR was used in diagnostics, but pan-AMDO-PCR was added later when more amdoparvoviruses were found from other species and the pan-AMDV-PCR might not be able to detect all of those.

2.2 PCR validation

Plasmids containing the PCR products were prepared to be used as quantitation controls by purifying PCR products amplified from a sample of an AMDV positive mink (MN590274-MN590276) with GeneJET PCR purification kit (Thermo scientific), cloning them into PGEM-T Easy –vector (Promega), and isolating plasmids with GeneJET Plasmid Miniprep kit (Thermo Scientific) according to manufacturers' instructions. Correctness of the sequences was confirmed by Sanger sequencing using primers pUC/M13 Forward (24mer) and pUC/M13 Reverse (22mer) and plasmid concentrations were measured with NanoDrop.

Analytical sensitivities were determined with plasmid dilution series containing 1000, 100, 50, 20, 10, 5, 1, and 0 copies/reaction and five parallel reactions. One parallel reaction containing 4 µl of DNA from AMDV-negative mink was also included to see if host DNA influences sensitivity. Specificities were tested with a set of 46 samples (samples 1-46, Table 1) that had been collected for diagnostic purposes between 2016 and 2019 and DNA was isolated from spleen with NucleoSpin Tissue –kit (Macherey-Nagel). Samples 26-34 and 39-46 were freshly isolated samples that had not yet been tested. Samples 1-25 and 35-38 were older samples that had earlier been diagnosed as positive but had gone through a varying number of freeze-and-thaw-cycles afterwards. A panel of 37 DNA-samples (samples 47-83, Table 2) from stool of mink, foxes and one raccoon with canine parvovirus (CPV), Feline panleukopeniavirus (FPLV), or mink enteritis virus (MEV) was also tested to ensure that PCRs are not cross reacting with other parvoviruses. PCRs that gave contradictory results were repeated. Positive, negative and unspecific results of pan-AMDV and pan-AMDO –PCRs were determined by looking at the dissociation curve, by running samples in a 2% agarose gel or by Sanger sequencing. Selected PCR products were purified for sequencing by adding 0.5 µl of Exonuclease I and 1 µl of FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) to 5 µl of PCR reaction and incubating them at 37 °C for 45 min and 85 °C for 15 min.

2.3 Data analysis

Diagnostic sensitivities and specificities were calculated with MedCalc Software

(https://www.medcalc.org/calc/diagnostic_test.php). Pan-AMDV sequences from samples 47-51 and pan-AMDO-sequences from samples 47, 48, and 52 were deposited to GenBank under accession numbers MN590277-MN590284. Partial or complete coding regions of samples 1-15, 19-21, and 36-37 have already been sequenced in our earlier study (accession numbers shown in Table 1). To demonstrate the relationships between these strains, other strains circulating in Finland, and selected global strains, sequences were aligned with MEGA6 (31) using ClustalW and maximum likelihood -trees were constructed using Hasegawa Kishino Yano model (HKY+G) that was an estimated best fitting evolutionary model for both three. Raccoon dog amdovirus (RFAV-HS-R) was used as an outgroup (32).

3. Results

The lowest copy number that gave positive results with all five plasmid replicates was 20 copies/reaction for all three PCRs. In most cases, adding mink DNA caused no decrease or only a small decrease (less than 1 cycle) on Ct values.

Nine diagnostic samples were negative (38-46) and 34 were positive with all three PCRs (1-34). Three samples produced contradictory results (35-37) and one sample (38) that had been positive in initial diagnostics was now negative. Seven out of eleven negative samples had unspecific products with pan-AMDO-PCR. PCR results with Ct values (fluorescence threshold 0.04) are given in Table 1. Ct values of samples that were positive with all three PCRs are compared in Fig. 1.

Stool samples of CPV-, MEV-, or FPLV-positive mink, foxes and finnraccoon were tested to detect possible cross-reactivity with other parvoviruses (Table 2). Two mink samples (47 and 48) were positive and 31 samples (53-83) negative with all three PCRs. Four fox samples gave contradictory results (49-52). Five samples had unspecific products with pan-AMDV-PCR and 29 with pan-AMDO-PCR based on Sanger sequencing. No cross-reactivity with other parvoviruses was detected in any of the PCRs.

Diagnostic sensitivities and specificities were calculated based on the results of the first PCR round of spleen samples and spleen and stool samples combined (Table 3). Samples that were negative in all three PCRs were considered negative, samples that were positive with at least one of the PCRs were considered positive, and samples that gave fluorescence signal but proved to be negative based on further studies were considered false positive.

Based on the phylogenetic analysis of pan-AMDV- and pan-AMDO-PCR sequences (Fig. 2), mink strains are similar to the other Finnish strains. Fox strains 50 and 51 were similar to the mink strain 48 from the same farm and fox strain 49 appeared closest to the previously published Finnish and Chinese strains in the phylogenetic tree.

4. Discussion

In this study, we set up a probe-based AMDV-PCR and compared that to other two PCRs currently used in diagnostics in Finland. Serological methods measuring IgG-antibody responses to AMDV with ELISA are the main protocol to diagnose AMDV, as hundreds of thousands of samples need to be annually screened in Finland (26,27). However, PCR is needed to confirm the results in uncertain cases and in cases where the ELISA-positive result comes from a clean farm. PCR is also needed to find out if an animal is either transiently or persistently infected and thus likely shedding the virus since seropositive result does not necessarily mean current infection.

All three PCRs had similar detection limits (20 copies/reaction) based on plasmid dilution series. These detection limits are also applicable to spleen samples commonly used for diagnostics, as mink DNA did not have a significant inhibitory effect. When mink DNA was present, pan-AMDO-PCR gave a signal even with

zero plasmid copies probably due to the attachment of the primers into the host genome. None of the PCRs cross-reacted to produce positive signal with other tested parvoviruses.

All PCRs gave the same results for 42 out of 46 spleen samples and 33 out of 37 stool samples and contradictory results for three spleen samples and four stool samples. Ct values seem to be slightly higher in probe-PCR than they are in two other PCRs (Fig. 1) but because samples have been frozen between PCRs, values are not directly comparable. Ct values of samples with similar results were generally low (Tables 1 and 2); only three of them had the Ct value above 35 with NS1-probe-PCR. Contradictory results, which all had Ct values above 30, can probably be explained by several freeze-and-thaw cycles and by the fact that samples had low viral loads to begin with. Another possible explanation is that only 2 µl of isolated template DNA was used in NS1-probe-PCR and 5 µl in other two PCRs. To test this, we run NS1-probe-PCR again for samples with contradictory results with 5 µl of template, but the results were the same as with 2 µl (data not shown) indicating that smaller amount of template DNA did not decrease sensitivity. Even though high genetic variability can be a problem when designing primers for diagnostic PCRs (30), these false negatives were most likely caused by too low amount of AMDV-DNA in the sample, not mismatches with the primers. Many of the samples in the panel have earlier been sequenced and are known to have very diverse sequences. Based on these results, there is no difference in the ability of these PCRs to amplify the AMDV-strains currently circulating in mink in Finland. Also, it is important to avoid freeze-and-thaw cycles in diagnostics to avoid false negative results.

AMDV antibodies have earlier been found in foxes (33). Here, we detected AMDV DNA in stool of four foxes. Sequences from foxes and mink of farm 43 were similar, and another fox strain (49) also grouped together with mink strains. Results might indicate that foxes can be infected with the same AMDV-strains as mink and even secrete AMDV in stool. This, however, needs further studies since the same farm has the virus circulating in mink and the chance of contamination during sampling cannot be fully excluded. Results from fox stool samples were inconsistent and the applicability of these PCRs to foxes cannot be fully evaluated due to the small sample size. However, well evaluated diagnostics AMDV-tests for foxes are rarely needed and further studies are required to see if there would be need for one.

When only spleen samples were used in calculations, NS1-probe-PCR had slightly higher diagnostic sensitivity (97.3%) than other two PCRs (94.6%). When also stool samples were taken into account, sensitivity of the NS1-probe-PCR (90.7%) was slightly lower than that of the pan-AMDV-PCR (93.0%). However, all the false negative results from stool samples were from foxes and the results from mink were consistent with those of other two PCRs. The sensitivity calculated using mink spleen samples is more relevant to evaluate the diagnostic applicability of these PCRs since diagnostics in Finland is usually performed from mink spleen. The estimated specificity of the NS1-probe-PCR was 88.9%, which is due to

one sample that gave signal during the last cycle but was considered negative. If this sample is excluded, the specificity is 100%. This sample was weak positive in initial diagnostics so the possibility of the signal being a very weak positive result cannot be ruled out either. It should also be noted, that the amount of negative spleen samples was too low to give an exact number about specificity. Specificity of the NS1-probe PCR using stool samples seemed excellent compared to other two PCRs that both gave false positives which turned out to be bacterial or host DNA in sequencing. Especially pan-AMDO-PCR seemed to work poorly with stool samples. Although using a non-invasive (i.e. stool) sample would sound ideal, it would not be reliable in diagnostics because AMDV is not always secreted in feces. However, because of good specificity, the NS1-probe-PCR is a good protocol to screen stool samples for research purposes.

In summary, all three PCRs are able to amplify diverse AMDV-strains, which is one of the requirements for a good diagnostic PCR. Other requirements include good specificity and sensitivity to avoid false positive and false negative results, and the results should be easy to interpret. Based on this study and our earlier experience, pan-AMDV- and pan-AMDO-PCRs are very sensitive, but due to lower specificity, they often require confirmation by gel electrophoresis or sequencing, prolonging the time to reach the final result. Here, we showed that adding a probe increased the specificity significantly. All PCRs also showed similar analytic and diagnostic sensitivities. AMDO-PCR was designed to have a good sensitivity and ability to amplify other amdoparvoviruses, but it has much lower specificity than other two PCRs. It is well suitable in cases where virus strains are expected to be exceptionally diverse, for example, when studying species other than mink. Probe-PCR is the most practical out of the three, since it is less time consuming and enables faster results.

5. Conclusions

We successfully set up a novel probe-based AMDV-PCR and validated that and other two PCRs currently used in diagnostics in Finland. We also found AMDV-DNA from fox stool, indicating that foxes might also secrete AMDV. However, further studies are needed to understand the significance of AMDV infections in foxes, since the possibility of contamination could not be fully excluded.

NS1-probe-PCR showed similar or better analytic and diagnostic sensitivity as compared to the other two PCRs as well as a good specificity, thus establishing it as a promising tool for research and diagnostic purposes for AMDV, a major disease of mink.

Competing interests

Declarations of interest: none

Acknowledgments

We thank Johanna Martikainen for technical assistance and Anna Knuuttila for her contribution in setting up pan-AMDV- and pan-AMDO-PCRs. We also thank Fin Furlab and the farms for providing samples.

This study was supported by the Helve Foundation and Finnish Veterinary Foundation grants.

References

- (1) Gorham JR, Leader RW, Henson JB. The Experimental Transmission of a Virus Causing Hypergammaglobulinemia in Mink: Sources and Modes of Infection. *J Infect Dis* 1964 Oct;114:341-345.
- (2) Farid AH, Hussain I, Arju I. Detection of Aleutian mink disease virus DNA and antiviral antibodies in American mink (*Neovison vison*) 10 days postinoculation. *J Vet Diagn Invest* 2015 May;27(3):287-294.
- (3) Jensen TH, Hammer AS, Chriel M. Monitoring chronic infection with a field strain of Aleutian mink disease virus. *Vet Microbiol* 2014 Jan 31;168(2-4):420-427.
- (4) Gorham J, Henson J, Crawford T, Padgett G. The epizootiology of Aleutian disease. In: Kimberlain R, editor. *Slow virus diseases of animals and man*, Frontiers of Biology Amsterdam: North-Holland Publishing Co; 1976. p. 135-158.
- (5) Porter DD, Larsen AE, Porter HG. Reduced severity of lesions in mink infected transplacentally with Aleutian disease virus. *J Immunol* 1977 Sep;119(3):872-876.
- (6) Broll S, Alexandersen S. Investigation of the pathogenesis of transplacental transmission of Aleutian mink disease parvovirus in experimentally infected mink. *J Virol* 1996 Mar;70(3):1455-1466.
- (7) Bloom ME, Kanno H, Mori S, Wolfinbarger JB. Aleutian mink disease: puzzles and paradigms. *Infect Agents Dis* 1994 Dec;3(6):279-301.
- (8) Alexandersen S. Acute interstitial pneumonia in mink kits: experimental reproduction of the disease. *Vet Pathol* 1986 Sep;23(5):579-588.
- (9) Hartsough GR, Gorham JR. Aleutian disease in mink. *Nat. Fur News*. 1956(28):10-11.
- (10) Aasted B. Aleutian disease of mink. *Virology and immunology. Acta Pathol Microbiol Immunol Scand Suppl* 1985;287:1-47.
- (11) Knuuttila A, Aaltonen K, Virtala AM, Henttonen H, Isomursu M, Leimann A, et al. Aleutian mink disease virus in free-ranging mustelids in Finland - a cross-sectional epidemiological and phylogenetic study. *J Gen Virol* 2015 Jun;96(Pt 6):1423-1435.
- (12) Farid AH. Aleutian mink disease virus in furbearing mammals in Nova Scotia, Canada. *Acta Vet Scand* 2013 Feb 8;55:10-0147-55-10.
- (13) Fournier-Chambrillon C, Aasted B, Perrot A, Pontier D, Sauvage F, Artois M, et al. Antibodies to Aleutian mink disease parvovirus in free-ranging European mink (*Mustela lutreola*) and other small carnivores from southwestern France. *J Wildl Dis* 2004 Jul;40(3):394-402.
- (14) Murakami M, Matsuba C, Une Y, Nomura Y, Fujitani H. Nucleotide sequence and polymerase chain reaction/restriction fragment length polymorphism analyses of Aleutian disease virus in ferrets in Japan. *J Vet Diagn Invest* 2001 Jul;13(4):337-340.
- (15) Shahrabadi MS, Cho HJ, Marusyk RG. Characterization of the protein and nucleic acid of Aleutian disease virus. *J Virol* 1977 Aug;23(2):353-362.

- (16) Bloom ME, Race RE, Wolfinbarger JB. Characterization of Aleutian disease virus as a parvovirus. *J Virol* 1980 Sep;35(3):836-843.
- (17) Cotmore SF, Agbandje-McKenna M, Chiorini JA, Mukha DV, Pintel DJ, Qiu J, et al. The family Parvoviridae. *Arch Virol* 2014 May;159(5):1239-1247.
- (18) Bloom ME, Alexandersen S, Perryman S, Lechner D, Wolfinbarger JB. Nucleotide sequence and genomic organization of Aleutian mink disease parvovirus (ADV): sequence comparisons between a nonpathogenic and a pathogenic strain of ADV. *J Virol* 1988 Aug;62(8):2903-2915.
- (19) Bloom ME, Alexandersen S, Garon CF, Mori S, Wei W, Perryman S, et al. Nucleotide sequence of the 5'-terminal palindrome of Aleutian mink disease parvovirus and construction of an infectious molecular clone. *J Virol* 1990 Jul;64(7):3551-3556.
- (20) Qiu J, Cheng F, Burger LR, Pintel D. The transcription profile of Aleutian mink disease virus in CRFK cells is generated by alternative processing of pre-mRNAs produced from a single promoter. *J Virol* 2006 Jan;80(2):654-662.
- (21) Qiu J, Cheng F, Pintel D. The abundant R2 mRNA generated by aleutian mink disease parvovirus is tricistronic, encoding NS2, VP1, and VP2. *J Virol* 2007 Jul;81(13):6993-7000.
- (22) Huang Q, Luo Y, Cheng F, Best S, Bloom M, Qiu J. Molecular characterization of the small nonstructural proteins of parvovirus Aleutian mink disease virus (AMDV) during infection. *Virology* 2014(452-453):23-31.
- (23) Hagberg EE, Pedersen AG, Larsen LE, Krarup A. Evolutionary analysis of whole-genome sequences confirms inter-farm transmission of Aleutian mink disease virus. *J Gen Virol* 2017 Jun;98(6):1360-1371.
- (24) Aasted B, Alexandersen S, Christensen J. Vaccination with Aleutian mink disease parvovirus (AMDV) capsid proteins enhances disease, while vaccination with the major non-structural AMDV protein causes partial protection from disease. *Vaccine* 1998 Jul;16(11-12):1158-1165.
- (25) Castelruiz Y, Blixenkrone-Moller M, Aasted B. DNA vaccination with the Aleutian mink disease virus NS1 gene confers partial protection against disease. *Vaccine* 2005 Jan 26;23(10):1225-1231.
- (26) Knuuttila A, Aronen P, Saarinen A, Vapalahti O. Development and evaluation of an enzyme-linked immunosorbent assay based on recombinant VP2 capsids for the detection of antibodies to Aleutian mink disease virus. *Clin Vaccine Immunol* 2009 Sep;16(9):1360-1365.
- (27) Knuuttila A, Aronen P, Eerola M, Gardner IA, Virtala AM, Vapalahti O. Validation of an automated ELISA system for detection of antibodies to Aleutian mink disease virus using blood samples collected in filter paper strips. *Virol J* 2014 Aug 8;11:141-422X-11-141.
- (28) Cho HJ, Ingram DG. Antigen and Antibody in Aleutian Disease in Mink. I. Precipitation Reaction by Agar-Gel Electrophoresis. *The journal of immunology* 1972;108:555-557.
- (29) Jensen TH, Christensen LS, Chriel M, Uttenthal A, Hammer AS. Implementation and validation of a sensitive PCR detection method in the eradication campaign against Aleutian mink disease virus. *J Virol Methods* 2011 Jan;171(1):81-85.
- (30) Virtanen J, Smura T, Aaltonen K, Moisander-Jylha AM, Knuuttila A, Vapalahti O, et al. Co-circulation of highly diverse Aleutian mink disease virus strains in Finland. *J Gen Virol* 2019 Feb;100(2):227-236.

- (31) Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 2013 Dec;30(12):2725-2729.
- (32) Shao XQ, Wen YJ, Ba HX, Zhang XT, Yue ZG, Wang KJ, et al. Novel amdoparvovirus infecting farmed raccoon dogs and arctic foxes. *Emerg Infect Dis* 2014 Dec;20(12):2085-2088.
- (33) Ingram DG, Cho HJ. Aleutian disease in mink: virology, immunology and pathogenesis. *J Rheumatol* 1974 Mar;1(1):74-92.

Fig. 1. Ct values of samples that were positive with all PCRs sorted from smallest to largest based on NS1-Probe-PCR.

Table 1

PCR results and Ct values of spleen samples with fluorescence threshold of 0.04. Ct values are reported even if no AMDV sequence was detected by Sanger sequencing. In these cases, results are considered negative. If two results and Ct values are reported, PCR was performed twice.

Id	Farm id	Species	Pan-AMDV	Ct value	Pan-AMDO	Ct value	NS1-probe	Ct value	GenBank accession no.
1	1	mink	+	31.66	+	31.7	+	35.83	MG821275, MG821295
2	2	mink	+	14.9	+	18.42	+	17.64	MG821270, MG821291
3	3	mink	+	32.6	+	32.38	+	35.09	MG821274, MG821294
4	4	mink	+	16.12	+	16.72	+	21.57	MG821273, MG821293
5	5	mink	+	31.38	+	28.12	+	27.76	MG821245
6	6	mink	+	23.99	+	29.96	+	30.71	MG821242
7	7	mink	+	29.81	+	27.59	+	34	MG821244
8	7	mink	+	18.97	+	17.7	+	22.03	MG821243
9	8	mink	+	22.32	+	21.85	+	29.22	MG821279, MG821299
10	9	mink	+	28.54	+	29.29	+	31.05	MG821267
11	2	mink	+	14.79	+	13.19	+	16.65	MG821266
12	8	mink	+	16.32	+	15.53	+	21.31	MG821278, MG821298
13	10	mink	+	20.18	+	19.72	+	26.35	MG821234
14	11	mink	+	17.82	+	17.11	+	21.48	MG821241

15	12	mink	+	17.45	+	15.63	+	22.54	MG821238
16	13	mink	+	31.21	+	27.99	+	25.72	
17	14	mink	+	22.76	+	21.12	+	27.32	
18	15	mink	+	22.17	+	22.81	+	24.24	MG821265
19	16	mink	+	21.01	+	17.87	+	21.28	
20	17	mink	+	21.79	+	21.33	+	26	
21	17	mink	+	15.95	+	15.26	+	21.09	MG821235
22	18	mink	+	33.08	+	24.25	+	35.49	
23	19	mink	+	25.35	+	31.61	+	22.8	
24	20	mink	+	32.28	+	26.21	+	28.23	MG821236
25	21	mink	+	29.37	+	28.58	+	31.51	
26	22	mink	+	19.27	+	21.21	+	26.28	
27	23	mink	+	14.7	+	15.14	+	21.01	MG821235
28	24	mink	+	16.8	+	16.16	+	18.3	
29	25	mink	+	18.43	+	18.84	+	25.09	
30	26	mink	+	18.34	+	19.61	+	21.36	MG821272, MG821292
31	27	mink	+	28.52	+	29.4	+	27.12	
32	28	mink	+	16.57	+	15.87	+	20.82	
33	29	mink	+	17.96	+	16.89	+	19.75	MG821271
34	30	mink	+	30.19	+	28.33	+	34.36	
35	31	mink	-/-	no ct/no ct	-/-	32.72/35.81	+/-	41.98/no ct	
36	32	mink	+/+	35.89/37.08	-/-	31.88/36.81	-/+	no ct/38.88	MG821271
37	33	mink	-/-	no ct/38.81	+/+	32.73/32.11	+/+	39.49/41.12	
38	1	mink	-/-	no ct/no ct	-/-	33.33/35.3	-/-	44.03/no ct	
39	34	mink	-	no ct	-	no ct	-	no ct	MG821271
40	35	mink	-	no ct	-	no ct	-	no ct	
41	36	mink	-	no ct	-	36.61	-	no ct	
42	37	mink	-	no ct	-	37.95	-	no ct	MG821271
43	38	mink	-	no ct	-	31.51	-	no ct	
44	39	mink	-	no ct	-	no ct	-	no ct	
45	40	mink	-	no ct	-	no ct	-	no ct	MG821271
46	41	mink	-	no ct	-	32.37	-	no ct	

Table 2

PCR results and Ct values of stool samples with fluorescence threshold of 0.04. Ct values are reported even if no AMDV sequence was detected by Sanger sequencing. In these cases, results are considered negative.

If two results and Ct values are reported, PCR was performed twice.

Id	Farm id	Species	Pan-AMDV	Ct value	Pan-AMDO	Ct value	NS1-probe	Ct value	GenBank accession no.
47	42	mink	+	34.25	+	30.37	+	39.93	MN590278, MN590282
48	43	mink	+	16.41	+	15.54	+	24.38	MN590279, MN590283
49	44	fox	+/-	33.77/no ct	-/-	no ct/no ct	+/-	35.24/37.61	MN590277
50	43	fox	+/-	34.89/no ct	-/-	32.55/34	-/-	no ct/no ct	MN590280

51	43	fox	+/-	33.61/37.78	-/-	32.15/35.26	-/-	no ct/no ct	MN590281
52	43	fox	-/-	no ct/no ct	+/-	32.85/35.85	-/-	no ct/no ct	MN590284
53	45	fox	-	no ct	-	34.19	-	no ct	
54	45	fox	-	no ct	-	35.59	-	no ct	
55	46	fox	-	no ct	-	34.41	-	no ct	
56	46	fox	-	no ct	-	39.53	-	no ct	
57	44	fox	-	no ct	-	32.82	-	no ct	
58	44	fox	-	no ct	-	38.83	-	no ct	
59	47	fox	-	no ct	-	36.55	-	no ct	
60	47	fox	-	no ct	-	37.51	-	no ct	
61	47	fox	-	37.88	-	38.04	-	no ct	
62	47	fox	-	no ct	-	37.41	-	no ct	
63	48	fox	-	33.84	-	36.52	-	no ct	
64	48	fox	-	37.62	-	31.24	-	no ct	
65	45	mink	-	no ct	-	no ct	-	no ct	
66	45	mink	-	no ct	-	35.42	-	no ct	
67		raccoon	-	no ct	-	35.96	-	no ct	
68	43	mink	-	no ct	-	35.11	-	no ct	
69	43	mink	-	no ct	-	no ct	-	no ct	
70	43	mink	-	no ct	-	33.51	-	no ct	
71	43	fox	-	no ct	-	39.3	-	no ct	
72	43	fox	-	no ct	-	33.43	-	no ct	
73	49	fox	-	no ct	-	34.05	-	no ct	
74	49	fox	-	no ct	-	34.89	-	no ct	
75	49	fox	-	no ct	-	38.01	-	no ct	
76	49	fox	-	no ct	-	33.66	-	no ct	
77	49	fox	-	35.96	-	35.15	-	no ct	
78	49	fox	-	no ct	-	33.89	-	no ct	
79	50	fox	-	no ct	-	33.73	-	no ct	
80	50	fox	-	no ct	-	39.67	-	no ct	
81	50	fox	-	no ct	-	36.67	-	no ct	
82	50	fox	-	38.17	-	36.71	-	no ct	
83	50	fox	-	no ct	-	36.35	-	no ct	

Table 3

Specificities and sensitivities for spleen samples and all samples combined.

		NS1-probe-PCR		pan-AMDV-PCR		pan-AMDO-PCR	
		%	95% CI	%	95% CI	%	95% CI
Spleen samples	Specificity	88.9*	51.8-99.7	100.0	66.4-100.0	44.4	13.7-78.8
	Sensitivity	97.3	85.8-99.9	94.6	81.8-99.3	94.6	81.8-99.3
All samples	Specificity	97.5	86.8-99.9	87.5	73.2-95.8	15.0	5.7-29.8
	Sensitivity	90.7	77.9-97.4	93.0	80.9-98.5	88.4	74.9-96.1

* Specificity is 100% if unclear sample 38 is excluded

Fig. 2. Phylogenetic analysis of pan-AMDV (a) and pan-AMDO (b) sequences. Best fitting evolutionary models (HKY+G) were defined and trees constructed with MEGA6. Bootstrap values above 70 are shown next to the nodes.