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Aleutian Mink Disease Virus in the breeding environment in Poland and its place in the global epidemiology of AMDV

Marek Kowalczyk, Beata Horecka, Andrzej Jakubczak*

Institute of Biological Basis of Animal Production, University of Life Sciences in Lublin, Akademicka 13, 20-950, Lublin, Poland

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ABSTRACT

Aleutian mink disease (AMD) leads to an increase in mortality of animals and causes losses in mink farming. The study investigated the presence of AMDV in tissue and environmental samples from farmed mink in Poland, and selected samples were genetically characterized.

Blood, spleens and swabs from the breeding environment were collected on 27 farms in seven voivodeships in Poland (n = 250). DNA was isolated, amplified by PCR and subsequently subjected to sequencing to reveal information on the molecular epidemiology of the samples. A qPCR method was used to determine the viral load in test samples.

The presence of AMDV was confirmed in tissues and the farm environment on 26 of the 27 farms. The average viral load in spleens was 10^8 copies. The virus was also present in the blood (average - 10^5 copies) and the farm environment (average - 10^3 copies). Isolates from the West Pomeranian Voivodeship showed high similarity within the voivodeship (over 99%). Variants from the Lublin and Podlaskie Voivodeships differed 5% from any of the AMDV isolates present in the NCBI database. Isolates from the Greater Poland, Pomeranian, Podkarpackie and Lesser Poland Voivodeships formed heterogeneous clades, showing over 97% similarity to variants previously isolated in Poland, the Netherlands and Lithuania.

A high degree of genetic variation was identified among the majority of the samples, which indicates that AMDV has been introduced to Poland multiple times. However, the results within one area showed high identity between isolates, suggesting that one common ancestor was the source of these outbreaks.

1. Introduction

Viral diseases pose a serious threat to farming efficiency and animal welfare. In addition to animal diagnostics, monitoring of the sanitary condition of the farm environment and assessment of molecular variation in the etiological factors circulating between outbreak sites are of great importance both in disease prevention and in epidemiological studies. The high persistence of some infectious agents in the environment (Prieto et al., 2017, 2014) and the fact that deposition of the pathogen in the breeding area is conducive to the development of new pathways of its circulation, including anthropogenic ones (Cao et al., 2018), necessitates analysis of environmental samples in epidemiological studies. Molecular methods are increasingly used to track the flow of pathogens in the case of diseases in both humans (Sukhrie et al., 2011) and animals (Cottam et al., 2008).

AMDV belongs to the genus *Amdovirus* in the family *Parvoviridae*, whose representatives are highly persistent in the environment and resistant to both physical and chemical factors (Eterpi et al., 2009). It is a significant cause of reduced fertility in females on infected farms, due to an increase in the number of sterile females and abortions, as well as smaller litter size (Knuuttila et al., 2009; Leimann et al., 2015).

Early detection of AMDV is one of the most important measures aimed at eliminating the pathogen from farms. A technique enabling quantitative evaluation of the viral load in a test sample is Real-Time PCR (qPCR), which is used to amplify genetic material of the virus and simultaneously quantify the amount of specific DNA in the sample. The qPCR technique is highly sensitive and can also be used to detect the pathogen in environmental samples (Prieto et al., 2014), and thereby determine the efficiency of disinfection measures or eradication strategies. By supplementing the diagnostics with an additional sequencing step combined with bioinformatic analysis, the molecular epidemiology of AMDV in a given area can be assessed and the circulation of the pathogen between outbreak sites can be analysed (Jakubczak et al., 2016; Knuuttila et al., 2009; Persson et al., 2015; Wang et al., 2014).

Poland is one of the leading producers of mink skins, and AMD is becoming a significant and common problem (Jakubczak et al., 2016;

* Corresponding author at: University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland.
E-mail address: andrzej.jakubczak@up.lublin.pl (A. Jakubczak).

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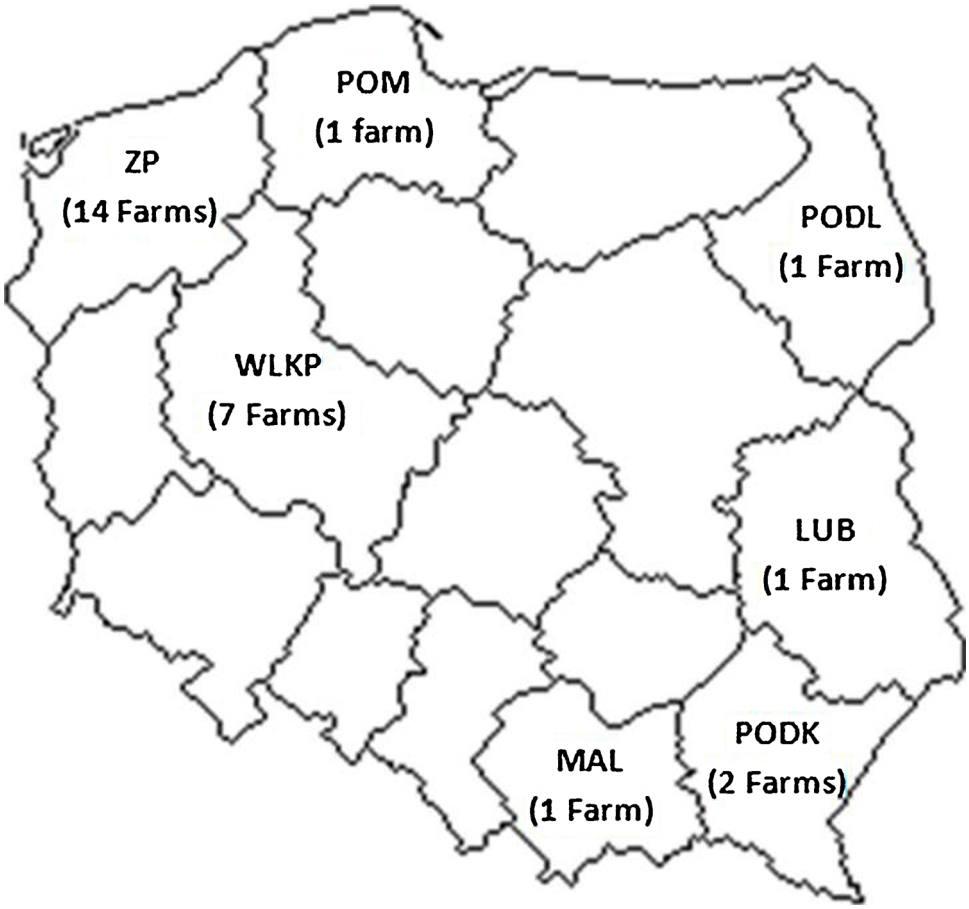

Fig. 1. Location of farms.

Kowalczyk et al., 2018; Reichert and Kostro, 2014a, b). The aim of the study was to evaluate the use of qPCR to diagnose Aleutian disease in tissues of infected animals and to identify AMDV in environmental samples. Furthermore, the viral load was determined and genetic analysis was performed to investigate molecular variation in the virus on Polish farms, taking into account the global AMDV epidemiology map.

2. Material and methods

2.1. Farms

Fourteen farms from the West Pomeranian Voivodeship, seven farms from the Greater Poland Voivodeship, two from the Podkarpackie Voivodeship and one each from the Lublin, Lesser Poland, Podlaskie and Pomeranian voivodeships were selected for the study (Fig. 1). Farms that showed positive results in CIEP tests were chosen for the analysis. Samples (n = 25) from a farm from the Greater Poland Voivodeship where CIEP tests had been negative from the start of the farm's operation were used as a negative control. More farms were selected from the West Pomeranian and Greater Poland Voivodeships due to the high concentration of production in these two regions, where more than 35% of Polish mink farms are located.

2.2. Sampling

The material for the tests comprised tissue (spleen n = 55), blood (n = 35), pooled blood samples (n = 35 pooled samples comprising 10 samples each, 350 blood samples in total), and samples from the environment of 12 farms (n = 125, including 25 samples from a negative control farm). In total 250 samples were analysed. Blood was collected into 2K-EDTA microcapillary tubes (Biomaxima, Lublin, Poland).

In order to propose a method allowing simultaneous examination of a large group of animals, the possibility of examining pooled samples was tested. Material from 350 animals was prepared, creating pooled samples comprising 10 samples each. Pooled samples were obtained by mixing 40 ul of blood from 10 individuals, and from that pooled sample 200 ul was collected and further treated as a single sample during DNA isolation. Pooling of 350 single samples resulted in 35 pooled samples.

Swabs from the farm environment were collected by wiping the surface with a sterile swab moistened with PBS and placing it in a transport medium. Environmental swabs from tested farms were taken from nesting boxes (n = 64), construction elements of the mink sheds (n = 15), farm equipment (n = 8), and the floor of the sheds (n = 6), as well as from staff lounges (n = 7) (supplementary material 1). Farm samples constituting the negative control were taken from the nesting boxes.

2.3. DNA isolation

DNA was isolated from the swabs, blood, pooled blood and spleens with a DNeasy Blood and Tissue kit (Qiagen Hilden, Germany). Tissue samples from several regions of the spleen were cut into small pieces (in total 10 mg). The tissue was homogenized with 200 ul ATL lysing buffer in a TissueLyser (Qiagen Hilden, Germany) apparatus (20 hz for 30 s) and incubated at 56 °C until lysis was complete. Further isolation steps were carried out according to the manufacturer's protocol. DNA was eluted with 150 ul of elution buffer.

In the case of blood, DNA was extracted from 200 ul of blood, and the manufacturer's protocol was followed. DNA was eluted with 100 ul of elution buffer.

Table 1
Locations of the farms, number of samples tested and sequenced, and number of pathogen variants obtained. Variants with the same lowercase letters are identical (100% similarity between sequences).

Location (voivodeship)	Farm	Number of samples tested by PCR	Number of sequenced samples	Variants of the virus
West Pomeranian	ZP1	4	3	ZP1A[b], ZP1B[a]
	ZP2	10	3	ZP2[a]
	ZP3	14	3	ZP3[a]
	ZP4	10	3	ZP4[a]
	ZP5	5	3	ZP5[a]
	ZP6	5	3	ZP6[a]
	ZP7	5	3	ZP7[a]
	ZP8	5	3	ZP8[a]
	ZP9	14	3	ZP9[a]
	ZP10	5	3	ZP10[a]
	ZP11	5	3	ZP11[a]
	ZP12	3	3	ZP12[a]
	ZP13	4	3	ZP13A[c], ZP13B[a]
	ZP14	3	3	ZP14[a]
Greater Poland	WLKP1	4	3	WLKP1A[d], WLKP1B[e]
	WLKP2	3	3	WLKP2A, WLKP2B[e], WLKP2C
	WLKP3	5	3	WLKP3
	WLKP4	10	3	WLKP4A, WLKP4B, WLKP4C[d]
	WLKP5	5	3	WLKP5
	WLKP6	10	3	WLKP6A, WLKP6B
Podkarpackie	PODK1	12	5	PODK1A, PODK1B
	PODK2	12	5	PODK2A, PODK2B, PODK2C, PODK2D
Lesser Poland	MAL	4	4	MAL1A, MAL1B
Podlaskie	PODL	6	4	PODL1A, PODL1B
Lublin	LUB	10	4	LUB1
Pomerania	POM	10	4	POM1A, POM1B
Total	**26**	**183**	**86**	**41**

Prior to isolation, the swabs were incubated with shaking for 2 h in 400 ul PBS at 37 °C, followed by isolation. After incubation, swabs with PBS were subjected to lysis in 400 ul AL buffer and 20 ul proteinase K at 56 °C with shaking for 1 h. Further isolation steps were performed according to the manufacturer's protocol. DNA was eluted with 100 ul of elution buffer.

2.4. Evaluation of the viral load in the tissues and farm environment by qPCR

DNA isolation and quantification by qPCR were performed for all samples. The qPCR method was also used to test samples from the farm constituting the negative control. Determination of the number of copies of the genetic material of the virus was carried out by qPCR using a commercial assay for Aleutian Disease Virus targeting part of the sequence encoding the NS1 protein (Gensig AIDV advanced Kit - PrimerDesign™ Ltd., Eastleigh UK) in an ABI Prism 7500 Fast apparatus (Applied Biosystems, Foster City, CA, USA). Quantitative determination of the average number of copies of viral DNA in each of the test materials was performed. Serial dilutions of 2×10^5 copies/µl, 2×10^4 copies/µl, 2×10^3 copies/µl, 2×10^2 copies/µl, 2×10^1 copies/µl and 2×10^0 copies/µl were used to prepare the standard curve. These dilutions were prepared using the positive control supplied with the kit.

2.5. PCR

PCR was performed for DNA samples isolated from all spleens and blood samples and for swabs in which the viral load was at least 10^2 copies (in total 183 samples from 26 farms). The reaction was carried out with a primer amplifying the sequence encoding a fragment of the NS1 protein (forward primer 5′ CATATTCACTGTTGCTTAGGTTA 3′ and reverse primer 5′ CGTTCTTTGTTAGTTAGGTTGTC 3′), previously used by Jensen et al. (2011). The reaction mixture contained 3 µl DNA and 1 U Taq polymerase AmpliTaq Gold 360 DNA Polymerase (Applied Biosystems, Foster City, CA, USA) in the manufacturer's buffer, adjusted to a final concentration of 2.5 mM $MgCl_2$, 0.8 mM of each dNTP, and 1.2 mM of each primer – 25 µL total volume. The reaction took place under the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 45 s, 54 °C for 45 s, 72 °C for 45 s, and 72 °C for 10 min in a Labcycler thermocycler (SensoQuest, Göttingen, Germany). The reaction products were separated on a 1% agarose gel with ethidium bromide at 80 V. Visualization and archiving of the gel was carried out in Scion Image software.

2.6. Sequencing and bioinformatic analysis

Following PCR, samples (n = 86) showing a positive result and yielding clear amplification products were selected for sequencing (Table 1). The samples were purified with an EPPiC Fast kit (A&A Biotechnology, Gdynia, Poland).and subjected to sequencing PCR with the same primers as in the standard reaction, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing PCR products were purified using the Exterminator kit (A&A Biotechnology, Gdynia, Poland). The purified samples were suspended in formamide, denatured, and then separated on an ABI PRISM 3100 Avant genetic analyser (Applied Biosystems, Foster City, CA, USA).

Sequencing results were assembled into contigs in DNA Baser software to obtain fragments of 305bp. Specificity was confirmed using the Blast application and sequences were compared with the NCBI bioinformatic database. Sequences obtained during the analyses were compared with sequences from the Genbank database in MEGA6 software (Tamura et al., 2013). The similarity between isolates was determined using Bioedit software. Analysis of polymorphisms and phylogenetic analysis were carried out in MEGA6. A phylogenetic tree was obtained by the Maximum Likelihood method based on the HKY model, with 1000 bootstrap replications. Sequences obtained during this study are available in GenBank with the accession numbers: MN175699-MN175739

2.7. Statistical analysis

The results of quantitative analysis were calculated by analysis of variance using the least squares method. The calculations were made

Table 2
Quantitative assessment of the viral load in the tissues and breeding environment of infected animals (*The number of AMDV copies in the spleen was statistically significantly higher than in the other types of material, at $p \leq 0.05$).

Type of material	Number of samples	Mean viral load	Minimum	Maximum
Tissue (spleen)	55	1.59E+08*	7.35E+02	2.21E+09
Blood	35	1.77E+05	1.41E+01	2.11E+06
Pooled blood	35	1.45E+04	2.34E+01	1.63E+05
Environmental swabs	100	2.49E+03	1.10E+01	8.20E+04

Table 3
Evaluation of the viral load in the environment of the farms (*The number of AMDV copies on farm 10 was statistically significantly higher than on the other farms, at $p \leq 0.05$).

Farm	Number of samples	Number infected (%)	Mean number of copies	Minimum	Maximum
1	8	4 (50%)	3.18E+02	1.87E+02	4.42E+02
2	10	5 (50%)	4.04E+03	1.15E+01	1.80E+04
3	8	4 (50%)	1.75E+03	4.54E+01	4.38E+03
4	20	18 (90%)	8.40E+02	6.50E+01	3.01E+03
5	6	5 (83%)	1.32E+02	2.26E+01	3.51E+02
6	3	2 (67%)	1.07E+02	5.65E+01	1.58E+02
7	10	9 (90%)	2.02E+03	9.95E+01	7.25E+03
8	5	2 (40%)	1.46E+03	2.29E+02	2.69E+03
9	5	3 (60%)	1.92E+02	5.23E+01	4.07E+02
10	10	9 (90%)	1.16E+04*	1.31E+01	8.20E+04
11	15	8 (53%)	1.46E+02	1.10E+01	6.60E+02
Total	**100**	**69 (69%)**	**2.06E+03**	**1.10E+01**	**8.20E+04**

using SAS statistical package (SAS Institute, Cary, NC, USA). Statistical significance was established as $p \leq 0.05$.

3. Results

The presence of AMDV genetic material was confirmed in each of the spleen and blood samples and in 69% of the swabs from farms with positive CIEP results and selected for quantitative evaluation. In the case of the farm samples used as a negative control, qPCR confirmed the lack of genetic material in each of the samples.

Quantification of the viral load in the tissues and farm environment showed the highest number of virus copies in the spleen (average of all positive spleens - 10^8 copies per reaction), with a minimum viral load in the spleen of over 700 copies (Table 2). In the case of blood, which is the principal diagnostic material for AMDV diagnosis, the average number of copies was 10^5. In the pooled samples, the number of copies of the virus was lower by one log.

On positive farms AMDV was also identified in the environment. The qPCR analysis showed that on average over 69% of the swabs tested positive, confirming the presence of AMDV in the breeding environment, with an average viral load of approximately 2000 copies per reaction (Table 3). The highest viral load was detected in the nesting boxes, construction elements of the sheds, and farm equipment (average over 10^3 copies). Genetic material was also detected on the floors of the sheds and in the staff lounges (supplementary 1).

The results of PCR with primers flanking the fragment encoding the NS1 protein indicated the presence of AMDV genetic material in each of the 183 samples. The specificity of the reaction was confirmed by sequencing and bioinformatic analysis of selected products. The samples showed more than 95% similarity to the AMDV sequences deposited in the NCBI database. The variants of the virus showed considerable variability between farms and relatively high similarity within farms. In the case of farms in the West Pomeranian Voivodeship (ZP), one variant of the virus was detected on twelve of the farms, while two variants of the virus, 99% similar to one another, were found on farms ZP1 and ZP13. Comparison of all isolates from this area indicated high homogeneity of the viral pool; the sequences obtained from all ZP farms were more than 99.5% similar to one another, and 14 of 16 isolates were 100% similar. Variants from the ZP group differed considerably from virus isolates from other parts of the country (differences ranging from 8.7% to 12.7%) (Table 4).

Variants from the Greater Poland Voivodeship (WLKP) showed much greater variation within the group. Twelve variants of the virus were distinguished on the six farms. Two groups of viruses circulating in this area were distinguished based on the polymorphisms between the isolates. The first group, WLKP I, comprises variants from farms WLKP1, WLKP2 and WLKP4. Farms WLKP5 and WLKP6 made up a second group, WLKP II, which showed over 96% similarity to WLKP I. The WLKP3 variant, which differed from both of these groups, was included in WLKP I based on the phylogenetic analysis. Isolates from the WLKP and ZP groups, despite the relatively small geographical distance between them, constitute two separate groups of the virus, differing by more than 10% within the NS1 protein coding sequence.

The largest number of tested farms were from the West Pomeranian and Greater Poland Voivodeships. Other regions of Poland were represented by fewer farms, but molecular polymorphism of AMDV was detected there as well (more than one variant of the virus was detected on each farm, except for farm LUB in the Lublin Voivodeship). Particularly high variation was noted on farm PODK2, where four closely related variants of the virus were detected (similarity between variants from 99% to 99.6%). High similarity of nearly 98% was observed between the Lesser Poland (MAL) variants and the Podkarpackie farms, PODKP1 and PODKP2. The isolates from farm POM, from the Pomeranian Voivodeship, showed similarity to variants from Greater Poland, especially to representatives of the WLKPI group, where the similarity exceeded 98.5%.

Phylogenetic analysis distinguished four main clades (Fig. 2). The first consisted of isolates from north-western Poland (variants from group ZP), showing low variation within the group. The second clade contained isolates from eastern Poland, formed by the two groups LUB and PODL. There was much greater variation in the third clade, so that two main subgroups were distinguished (IIIa, IIIb). The subgroup comprising the most variants, IIIa, was composed mainly of isolates

Table 4
Assessment of similarity between isolates.

	ZP								
ZP	**99.59%**	PODK							
PODK	87.28%	**98.36%**	MAL						
MAL	87.95%	97.77%	**98.00%**	WLKP					
WLKP	88.99%	94.40%	94.52%	**97.72%**	WLKP I				
WLKP I	89.12%	94.64%	94.71%	98.06%	**98.79%**	WLKP II			
WLKP II	88.57%	93.66%	93.95%	96.68%	96.14%	**99.10%**	LUB		
LUB	91.10%	91.73%	92.70%	93.41%	93.52%	93.07%	**100.00%**	PODL	
PODL	91.25%	92.33%	93.05%	93.63%	93.78%	93.17%	98.15%	**99.00%**	POM
POM	89.95%	94.32%	94.20%	98.01%	98.59%	96.28%	93.85%	94.20%	**99.60%**

Grey shade means the similarity of variants from the same region.

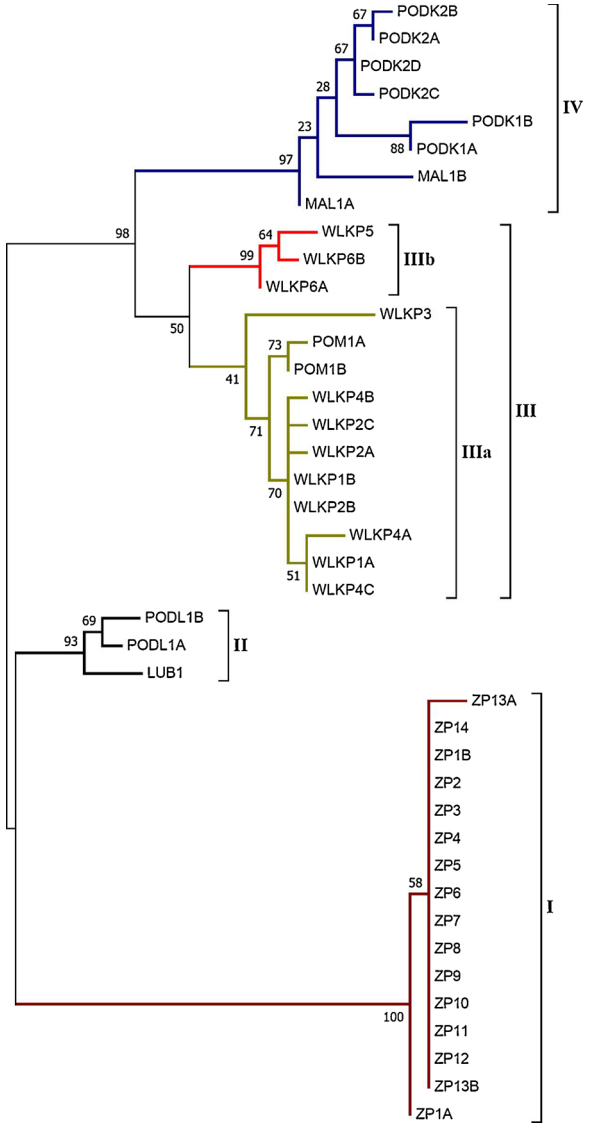

Fig. 2. Results of phylogenetic analysis of sequences of the AMDV virus circulating on Polish farms.

from Greater Poland and from Pomerania. Subgroup IIIb contained isolates from two Greater Poland farms that showed close phylogenetic relationships with IIIa representatives. Group IV comprised isolates from the Podkarpackie and Lesser Poland Voivodeships.

The ZP variants constituting Group I showed more than 99% similarity to variants isolated in Greece and the Netherlands in 2016 (Table 5). The sequences that showed the greatest similarity to representatives of the second group (over 94.7%) were obtained from samples isolated in Lithuania, Sweden and Italy. Isolates from the Greater Poland region, included in the IIIa subgroup, were about 99% similar to the AMDV variants isolated in Poland in 2016 and to isolates from the Netherlands. Variants from group IIIb were less similar to the sequences from the GenBank database. The sequences showing the highest similarity to the representatives of this group (over 97%) were isolates from the Netherlands. ADMV variants isolated from the Podkarpackie and Lesser Poland regions (group IV) were more than 97% similar to the sequences of the virus isolated in Poland and Lithuania.

4. Discussion

AMDV is a significant and common problem in Polish mink farming (Jakubczak et al., 2016; Kowalczyk et al., 2018; Reichert and Kostro, 2014b). The results of quantitative analysis by the qPCR method indicate that the most genetic material of the virus was in the spleen, largely due to the specific nature of the pathogen, which exhibits tropism for elements of the lymphatic system (Best and Bloom, 2005; Reichert and Kostro, 2014a). The determination of the amount of virus in the blood, as well as assessment of the possibility of pooling samples, suggest that the qPCR technique is a senstitive and promising tool for quick screening of an entire herd of animals for the presence of AMDV. Sample pooling protocols may be particularly useful in disease prevention and protection of farms from the risk of introducing AMDV together with new animal material. However, pooling of the samples may cause some of the viral load to be lost, as samples containing viral genetic material are diluted by those derived from healthy animals. In this case, potentially infected samples constitute only part of the total sample (dilution effects).

Prieto et al. (Prieto et al., 2017, 2014) used the qPCR method to confirm the high incidence of AMDV in the breeding environment, indicating possible pathways of transmission of the pathogen. Detection of the virus in tyre tracks and on workers' clothing indicates the important role of the anthropogenic factor in the spread of AMDV. Despite routine controls, intensive trade in animals fosters the spread of infectious diseases and the emergence of new disease outbreaks (Fevre et al., 2006). Our results also indicate deposition of the pathogen in the breeding environment of the farms, which makes them a potential reservoir as well as a medium conducive to the spread of disease, both within the farm and between farms.

The highest viral load was detected on surfaces with which the animals had direct contact. AMDV was present in swabs from nesting boxes, which seem to be the first site of deposition of viral material from infected animals. Positive results were also obtained in the case of the sheds (walls and roof), where the viral load was higher than in the nesting boxes. This may be due to the long-term exposition of these surfaces to AMDV. Nesting boxes are regularly cleaned and disinfected before new animals are introduced, so the risk of long-term deposition of a high load of the virus is limited. Detection of viral DNA on the floor and in the staff lounges supports the suggestion by Prieto et al. (2014) that AMDV may be spread by workers and visitors and that the pathogen may circulate within and most likely between farms.

The viral load detected in the farm environment by Prieto et al. (2017) ranged from 10^3 on the periphery of the farm to 10^7 in samples taken from cages. Our results, which indicate a lower viral load in the breeding environment (on average 10^3) as well as in the staff lounges (on average 10^2), are closer to the results from a previous study by Prieto et al. (2014), in which the authors detected viral DNA in numbers ranging from 10^2 to 10^5 copies in the cages and 10^3 copies in the meeting room. The difference may be due to a number of factors, such as the stage of infection, the sanitary condition of the farm, or preventive measures undertaken on the farm. Prieto et al.

The high persistence of AMDV in the environment, combined with the ease with which the virus is spread, significantly impedes efforts to eliminate the pathogen from farms (Christensen et al., 2011). The qPCR method is particularly useful in identifying pathways of virus flow and detecting its vectors. Research by Prieto et al. (2018) has indicated the possibility of transfer of the pathogen by flies, on farm workers' clothing, and by vehicles visiting infected farms (Prieto et al., 2014). Ryt-Hansen et al. (2017b) used polymorphism in the NS1 protein coding sequence to track the epidemiology of AMDV on Danish farms. Their analyses, which included testing of variation in the pathogen between outbreak sites as well as epidemiological data, indicated the potential for virus transmission together with deliveries of feed contaminated with infected components. We have confirmed that the virus was deposited in the environment of Polish farms, indicating that indirect transfer of the virus is highly likely.

Our analysis indicates high variation in the Aleutian disease virus in Poland, probably because the virus has been introduced multiple times from various sources. The variant from the West Pomeranian (ZP)

Table 5
Comparison of isolated variants with sequences from the NCBI database.

Country	I	II	III		IV
			IIIA	IIIB	
MG366646.1_Greece_2016	99.54%	91.20%	89.27%	88.57%	87.64%
MG366645.1_Greece_2016	99.54%	90.90%	88.95%	88.23%	87.76%
MG366644.1_Netherlands_2016	99.54%	90.90%	88.95%	88.23%	87.76%
MG366643.1_Netherlands_2016	98.54%	90.53%	88.14%	87.27%	87.45%
MG366642.1_Netherlands_2016	98.58%	90.90%	89.63%	88.23%	88.35%
MG366641.1_Netherlands_2016	98.28%	90.53%	88.07%	87.27%	87.45%
MG366640.1_Netherlands_2016	98.28%	90.53%	88.07%	87.27%	87.45%
MG366754.1_Lithuania_2016	89.49%	94.90%	95.97%	95.60%	95.71%
MG366753.1_Lithuania_2016	89.49%	94.90%	95.97%	95.60%	95.71%
MG366752.1_Lithuania_2016	89.49%	94.90%	95.97%	95.60%	95.71%
MG366755.1_Sweden_2016	90.10%	94.70%	95.12%	94.60%	95.61%
MG366711.1_Italy_2016	90.10%	94.70%	95.12%	94.70%	95.08%
MG366741.1_Poland_2016	89.49%	93.90%	99.30%	96.43%	94.91%
EF413730.1_Netherlands_2007	89.49%	93.60%	99.02%	96.77%	95.24%
MG366740.1_Poland_2016	89.11%	93.60%	98.96%	96.13%	94.58%
MG366749.1_Poland_2016	89.49%	94.60%	98.95%	96.43%	94.41%
MG366750.1_Netherlands_2016	89.49%	94.60%	98.89%	96.43%	94.50%
MG366738.1_Netherlands_2016	90.10%	93.60%	98.54%	96.77%	94.75%
MF074058.1_Denmark_2015	89.49%	93.50%	96.78%	96.43%	95.73%
MG366715.1_Netherlands_2016	88.80%	93.30%	96.35%	97.77%	94.50%
MG366717.1_Netherlands_2016	88.80%	93.30%	96.03%	97.43%	93.59%
MG366783.1_Poland_2016	87.50%	92.87%	94.55%	93.60%	98.11%
MG366780.1_Lithuania_2016	88.80%	93.80%	95.48%	95.23%	97.95%
MG366779.1_Lithuania_2016	88.80%	93.80%	95.48%	95.23%	97.95%
MG366782.1_Poland_2016	87.19%	91.87%	93.74%	92.63%	97.86%
MG366778.1_Lithuania_2016	88.11%	93.80%	94.87%	94.60%	97.53%
MG366781.1_Poland_2016	87.19%	92.60%	94.24%	93.30%	97.13%

Grey shade means the highest similarity of obtained isolates to the sequences from NCBI database.

group differed the most from the other isolates. The results of sequencing and bioinformatics analysis indicate the presence of similar variants of the virus on all tested farms in the West Pomeranian Voivodeship, which may be due to a common original source of infection for all farms or to the existence of common vectors for all farms leading to the spread of the pathogen from the primary outbreak of the disease.

The high similarity of the variant responsible for infection on farms from the ZP group to isolates from Greece and the Netherlands may indicate a common source of the virus that initiated the global flow of the pathogen or global flow of animals through trade. The virus, entering Poland from an infected source, may have been further disseminated by anthropogenic factors, leading to secondary local outbreaks of the disease. Variants from group ZP show very high similarity within the group and substantial dissimilarity to other Polish isolates, which may be due to the recent introduction of this variant to Poland.

AMDV, as a member of the Parvoviridae family, shows high molecular diversity. Analysis of the mutation rate of Canine Parvovirus type 2 (CPV-2) confirms that the rate of nucleotide substitution is more similar to that occurring in RNA viruses than in DNA viruses (Shackelton et al., 2005). Such high molecular polymorphism results in the emergence of new genetic variants of the pathogen (Faz et al., 2019; Kowalczyk et al., 2019). Sang et al., 2012 report that the mutation rate for the VP2 protein of AMDV is even higher than that of CPV-2. Therefore, more than one variant can be detected within a single farm or even a single host (Canuti 2016).

The homogeneity of the pathogen observed within group ZP may indicate that the virus had not yet produced more variants. The hypothesis of the recent appearance of this variant in Poland may also be supported by the course of the disease, characterized by a high mortality rate (up to 1% per day – Personal communication), which could limit the pathogen's variability.

Variants belonging to group III showed similarity to the virus isolated in 2016 in Poland, the Netherlands, and other countries. Isolates from group IV were similar to the variants detected in Poland and Lithuania. The relatively high dissimilarity of the group II variants to the NCBI database sequences may be indicative of the local and endemic nature of AMDV isolates from eastern Poland. Similar results have been obtained by Wang et al. (2016), who found that Chinese isolates were distributed in two groups and were unrelated to isolates from other countries. The finding that both local and imported ADMV variants are present in the Chinese mink population has also been confirmed by Sang et al. (2012).

Isolates from groups II, III and IV may be established in the Polish farmed mink population, as indicated both by their high genetic diversity and by the often persistent and sub-clinical course of infection. Modern mink breeding in Poland coincided with the political transformation. At the end of the 1990s, the first farms began to appear in Greater Poland, with breeding material imported from Sweden, Denmark, Finland and the United States. Animals from these countries were repeatedly imported into leading farms in Greater Poland. The current diversity of AMDV in the Greater Poland region can be explained by the diverse sources of biological material with which the pathogen may have been introduced. Long-term exposure of animals may promote increased tolerance to the disease. In the case of individuals undergoing selection or exposed to prolonged contact with the pathogen, the course of the disease may be milder (Farid and Ferns, 2017; Kowalczyk et al., 2018).

Ryt-Hansen et al., in their analysis of global variation of AMDV, also took into account Polish isolates from eight farms (Ryt-Hansen et al., 2017a). Comparison of sequences obtained by the researchers with our own results reveals that the majority of sequences (over 80%) obtained from Polish farms by the authors cited show high similarity to representatives of group III distinguished in our research (Supplementary

material 2). The researchers also obtained three sequences that formed a separate clade and differed by more than 10% from the sequences obtained in our study, which indicates a greater number of local outbreaks. Variants of the virus circulating in Poland largely exhibit local similarity, probably due to common pathways of infection for farms located in the same region. In the case of southern Poland, similar variants were detected in two voivodeships (group IV– Podkarpackie and Lesser Poland Voivodships). Farms separated by a considerable distance can be infected with similar variants of the virus, as confirmed by the high similarity between Pomeranian isolates and variants from Greater Poland, which may be due to a common origin of infected animals.

5. Conclusions

Aleutian disease remains a serious threat to mink farming around the world. Our results confirmed the presence of the virus both in animal tissues and in the breeding environment. The virus shows high variation, most likely because it has been introduced to farms multiple times. In addition to the variants detected in previous studies, established endemic variants as well as newly introduced variants are circulating in Poland, sharing high similarity with isolates present in the current global epidemiology of AMDV. Polish AMDV isolates seem to occur in local outbreaks within which the pathogen is transmitted by vectors that are common to infected farms. The combination of qPCR, sequencing and bioinformatic analysis is a valuable tool for identifying vectors and for studying the directions of the spread of AMDV, which in conjunction with veterinary history can help to eliminate the pathways of the spread of the virus and extinguish current outbreaks.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.virusres.2019.197665.

References

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. https://doi.org/10.1111/j.1439-0450.2005.00864.x.

Cao, J.M.D., Prieto, A., López, G., Fernández-Antonio, R., Díaz, P., López, C., Remesar, S., Díez-Baños, B., Fernández, G., 2018. Molecular assessment of visitor personal protective equipment contamination with the Aleutian mink disease virus and porcine circovirus-2 in mink and porcine farms. PLoS One 13 (8), e0203144. https://doi.org/10.1371/journal.pone.0203144.

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. https://doi.org/10.1016/j.vetmic.2010.10.016.

Cottam, E.M., Wadsworth, J., Shaw, A.E., Rowlands, R.J., Goatley, L., Maan, S., Maan, N.S., Mertens, P.P.C., Ebert, K., Li, Y., Ryan, E.D., Juleff, N., Ferris, N.P., Wilesmith, J.W., Haydon, D.T., King, D.P., Paton, D.J., Knowles, N.J., 2008. Transmission pathways of foot-and-mouth disease virus in the United Kingdom in 2007. PLoS Pathog. 4 (4), e1000050. https://doi.org/10.1371/journal.ppat.1000050.

Eterpi, M., McDonnell, G., Thomas, V., 2009. Disinfection efficacy against parvoviruses compared with reference viruses. J. Hosp. Infect. 73 (1), 64–70. https://doi.org/10.1016/j.jhin.2009.05.016.

Farid, A.H., Ferns, L.E., 2017. Reduced severity of histopathological lesions in mink selected for tolerance to Aleutian mink disease virus infection. Res. Vet. Sci. 111, 127–134. https://doi.org/10.1016/j.rvsc.2017.02.009.

Faz, M., Martínez, J.S., Gómez, L.B., Quijano-Hernández, I., Fajardo, R., Del Ángel-Caraza, J., 2019. Origin and genetic diversity of canine parvovirus 2c circulating in Mexico. Arch. Virol. 164 (2), 371–379. https://doi.org/10.1007/s00705-018-4072-7.

Fevre, E.M., Bronsvoort, B., Hamilton, K.A., Cleaveland, S., 2006. Animal movements and the spread of infectious diseases. Trends Microbiol. 14, 125–131. https://doi.org/10.1016/j.tim.2006.01.004.

Jakubczak, A., Kowalczyk, M., Kostro, K., Horecka, B., Jezewska-Witkowska, G., 2016. High molecular polymorphism of the hypervariable region in the VP2 gene of Aleutian mink disease virus. Acta Virol. 60, 354–360. https://doi.org/10.4149/av_2016_04_354.

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. https://doi.org/10.1016/j.jviromet.2010.10.004.

Knuuttila, A., Uzcategui, N., Kankkonen, J., Vapalahti, O., Kinnunen, P., 2009. Molecular epidemiology of Aleutian mink disease virus in Finland. Vet. Microbiol. 133, 229–238. https://doi.org/10.1016/j.vetmic.2008.07.003.

Kowalczyk, M., Jakubczak, A., Horecka, B., Kostro, K., 2018. A comparative molecular characterization of AMDV strains isolated from cases of clinical and subclinical infection. Virus Genes 54, 561–569. https://doi.org/10.1007/s11262-018-1576-x.

Kowalczyk, M., Majer-Dziedzic, B., Kostro, K., Szabelak, A., Ziętek, J., Gryzinska, M., Jakubczak, A., 2019. Diagnostics and genotyping of Canine parvovirus type 2 (CPV-2) from disease cases in south-eastern Poland. Acta Vet. (Beogr.) 69 (1), 32–46. https://doi.org/10.2478/acve-2019-0002.

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. https://doi.org/10.1016/j.virusres.2015.01.011.

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 10https://doi.org/10.1371/journal.pone.0122194. e0122194-e0122194.

Prieto, A., Diaz-Cao, J.M., Fernandez-Antonio, R., Panadero, R., Lopez-Lorenzo, G., Diaz, P., Perez-Creo, A., Morrondo, M.P., Fernandez, G., 2018. Lesser housefly (*Fannia canicularis*) as possible mechanical vector for Aleutian mink disease virus. Vet. Microbiol. 221, 90–93. https://doi.org/10.1016/j.vetmic.2018.05.024.

Prieto, A., Fernandez-Antonio, R., Diaz-Cao, J.M., Lopez, G., Diaz, P., Alonso, J.M., Morrondo, P., Fernandez, G., 2017. Distribution of Aleutian mink disease virus contamination in the environment of infected mink farms. Vet. Microbiol. 204, 59–63. https://doi.org/10.1016/j.vetmic.2017.04.013.

Prieto, A., Manuel Diaz-Cao, J., 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. https://doi.org/10.1016/j.vetmic.2014.07.024.

Reichert, M., Kostro, K., 2014a. Effect of persistent infection of mink with Aleutian mink disease virus on reproductive failure. Bull. Vet. Inst. Pulawy 58, 369–373. https://doi.org/10.2478/bvip-2014-0057.

Reichert, M., Kostro, K., 2014b. NS1 gene based molecular characteristics of Aleutian mink disease virus circulating in Poland. Bull. Vet. Inst. Pulawy 58, 187–191. https://doi.org/10.2478/bvip-2014-0028.

Ryt-Hansen, P., Hagberg, E.E., Chriel, M., Struve, T., Pedersen, A.G., Larsen, L.E., Hjulsager, C.K., 2017a. Global phylogenetic analysis of contemporary Aleutian Mink Disease Viruses (AMDVs). Virol. J. 14 (1), 231. https://doi.org/10.1186/s12985-017-0898-y.

Ryt-Hansen, P., Hjulsager, C.K., Hagberg, E.E., Chriel, M., Struve, T., Pedersen, A.G., Larsen, L.E., 2017b. Outbreak tracking of Aleutian mink disease virus (AMDV) using partial NS1 gene sequencing. Virol. J. 14 (1), 119. https://doi.org/10.1186/s12985-017-0786-5.

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 (1), 31–37. https://doi.org/10.1007/s11262-012-0734-9.

Shackelton, L.A., Parrish, C.R., Truyen, U., Holmes, E.C., 2005. High rate of viral evolution associated with the emergence of carnivore parvovirus. Proc. Natl. Acad. Sci. U. S. A. 102, 379–384. https://doi.org/10.1073/pnas.0406765102.

Sukhrie, F.H.A., Beersma, M.F.C., Wong, A., van der Veer, B., Vennema, H., Bogerman, J., Koopmans, M., 2011. Using molecular epidemiology to trace transmission of nosocomial norovirus infection. J. Clin. Microbiol. 49, 602–606. https://doi.org/10.1128/JCM.01443-10.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729. https://doi.org/10.1093/molbev/mst197.

Wang, Z., Wu, W., Hu, B., Zhang, H., Bai, X., Zhao, J., Zhang, L., Yan, X., 2014. Molecular epidemiology of Aleutian mink disease virus in China. Virus Res. 184, 14–19. https://doi.org/10.1016/j.virusres.2014.02.007.