Research Article

Serodiagnosis of Aleutian Disease Virus Infection in Mink – Short Term Stability and Long Term Consistency of Antibody Levels Measured by VP2 ELISA

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Abstract | Aleutian disease virus (ADV) infection in mink typically cause hypergammaglobulinemia, plasmacytosis and immune complex disease. Due to lack of success in eradicating Aleutian disease (AD) by stamping out, a new strategy for disease control with focus on evaluation of disease progression has been proposed. For estimation of antibodies in serum and dried blood samples collected from mink, a VP2 ELISA (enzyme-linked immunosorbent assay) system was recently evaluated. However, such estimations of disease progression are commonly based on single sampling occasions and it is essential to know if estimated antibody levels vary over a period of weeks or months. Therefore, the aim of this study was to evaluate the short term stability and the long term consistency of the antibody levels to Aleutian disease virus (ADV). When evaluated in the short term, i.e. between consecutive days and weeks, the estimated antibody levels were comparable and did not exceed the predefined equivalence interval based on the methodological variance for the VP2 ELISA. This confirms that the VP2 ELISA can be used for quantitatively estimating the ADV antibody levels even when the screening extends over a couple of weeks. For the long term consistency, a significantly higher mean ADV antibody level was recorded in February compared to in October. Therefore, if the VP2 ELISA should be used as a tool for selection of breeding females in a control program for AD, the optimal sampling time at which the ADV antibody levels best can predict the breeding performance of the female needs to be further evaluated.

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Introduction

Aleutian disease virus (ADV) typically induces hypergammaglobulinemia, plasmacytosis and immune complex disease in infected mink (Kenyon et al., 1963; Porter, 1986). Aleutian disease (AD) can range from mild to lethal disease and is associated with symptoms such as weight loss, reduced reproduction, polydipsia, polyuria, anemia and neurological symptoms (Hadlow et al., 1983; Paerson and Gorham, 1987; Bloom et al., 1994). Common to all forms of AD is the development of antibodies to both capsid and nonstructural ADV proteins (Bloom et al., 1975; Larsen and Porter, 1975; Bloom et al., 1982; Aasted and Bloom, 1984; Aasted et al., 1984; Porter et al., 1990). ADV infection in mink has a negative impact

on animal health and economy in the mink producing countries of the world. Due to lack of success in eradicating the disease by stamping out infected mink, a new strategy for control of AD has been suggested during the past decade which focuses on evaluation of AD progression rather than AD diagnosis (Themudo et al., 2011; Cepica et al., 2012; Andersson et al., 2015).

The new strategy is based on the idea that both the virus strain and different host factors influence the progression of the ADV infection. Thus, within an ADV infected herd, individual mink will differ in tolerance to the infection. As a consequence, infected mink will develop pathological lesions, clinical symptoms and fatal disease at different paces. Using a quantitative VP2 (virus capsid protein 2) ELISA (enzyme-linked immunosorbent assay) for ADV antibodies in serum or DBS (dried blood spot samples), the ratio of albumin: gamma globulins (A:γG) can be estimated and used as an indirect measurement of the disease progression (Henson et al., 1961; Cepica et al., 2012; Farid and Segervall, 2014; Andersson et al., 2015).

For the DBS VP2 ELISA, employing an optical density (OD_{450}) value of equal or more than 0.83 as the cut-off for diseased animals (representing A:γG < 5) and an OD_{450} value of less than 0.50 as the cut-off for healthy animals (representing A:γG > 8), mink could be categorized as diseased, ambiguous or healthy (Cepica et al., 2012; Andersson et al., 2015) (Figure 1).

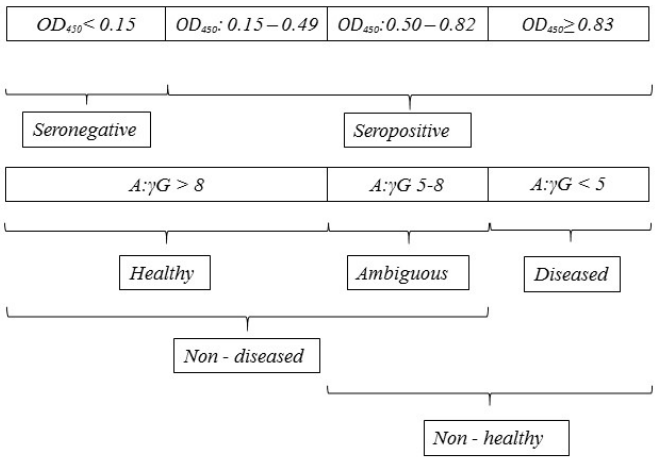

Figure 1: *The OD_{450} value as a predictor of ADV infection (seronegative/ seropositive) and AD progression defined by the albumin (A) : gamma globulin (γG) ratio in serum (A:γG)*

No studies of the consistency of antibody levels in mink have been published. However, studies in humans have indicated that immunoglobulin levels may vary physiologically with as much as 20% from one time to another (Veys et al., 1977; Meurman and Lehtonen, 1988). Therefore, it has been frequently discussed whether similar variations could be seen in mink as well. If the antibody levels vary physiologically, the evaluation of the disease progression in individual mink within an AD control program will be hard to interpret as it is based on the results from quantitative antibody detecting assays at a single sampling occasion. Thus, the aim of the first part of the study was to investigate if antibody levels against ADV in mink with different levels of AD symptoms vary from day to day or from week to week *i.e.* the short term stability.

Since an association between AD progression and litter size has been reported (Kangas, 1971; Hansen and Lund, 1988), another application of the quantitative ELISA could be to predict the breeding result. Traditionally, serological screening for AD is taking place during late autumn right before pelting. However, since the ADV antibody levels increase as the disease progresses and the serum gamma globulin levels vary throughout the winter (Kenyon et al., 1963; Bazeley, 1976; An et al., 1978), a change in ADV antibody level with time can be expected. Consequently, a more suitable time for screening and selection of breeding animals would be before mating, *i.e.* during the early spring (Bazeley, 1976; Bloom et al., 1994). Though, for disease control purposes there is an incitement of early culling, *i.e.* during late autumn. In order to investigate if the decision to cull the mink, depending on serological screening for AD status, would differ depending on time of screening (autumn or spring), the aim of the second part was to compare the ADV antibody levels as well as AD progression estimations in October and in February, *i.e.* the long term consistency.

Materials and Methods

Collection of blood samples

Part 1 - short term stability: 120 mink from two herds with different levels of clinical symptoms of AD were allocated into two different test groups encompassing 60 mink in each group (30 mink from each herd). The first group was sampled either day 1 and day 2 or day 1 and day 3, and the second group was sampled day

1, day 8 and day 15. In all groups, an equal number of mink from three different non-Aleutian color types was sampled in order to avoid coat color associated bias. Blood was sampled through toe nail clips into capillary tubes without additive, and stored at +2-8°C until transport to the laboratory where the blood samples were centrifuged at 850 x g and stored in -20°C until analysis.

Part 2 – long term consistency: Data in this part originated from routine analysis of blood samples from two consecutive screenings (October and February) of 350 white juvenile mink from one mink herd with clinical symptoms of chronic AD. In these samplings, the blood was collected as DBS through toe nail clip onto filter paper (Munktell TFN, VWR International AB, Stockholm, Sweden) and air-dried at room temperature (RT). The filter papers were placed separately inside envelopes, transported to the laboratory and stored at RT until analysis. The day before the analysis, the DBS were punched manually to a size of 25 mm^2 and eluted in elution buffer as previously described (Andersson et al., 2015).

Detection of antibodies to ADV

Part I - short term stability: Antibodies to ADV in the serum samples were estimated by an ELISA previously described (Andersson and Wallgren, 2013). In brief, 96-well immunoplates (Nunc Maxisorp, VWR International AB, Stockholm, Sweden) were coated with ADV VP2 antigen (Finnish Fur Breeder´s association, Vaasa, Finland) overnight, serum samples were diluted 1:200 and added to the plate and incubated for 60 min at RT. The plates were incubated together with reagent solution (peroxidase-conjugated goat anti-cat IgG, Fisher Scientific, Gothenburg, Sweden) and substrate (3,3′,5,5′-tetramethyl benzidine, Svanova, Uppsala, Sweden) before the reaction was stopped and the optical density (OD) was measured at 450 nm (Sunrise ELISA micro plate reader, Tecan Nordic AB, Mölndal, Sweden). The mean OD of the blank wells was subtracted from each result. Reference sera (negative and positive) were included on each plate and the results obtained were adjusted to a value of OD_{450} = 1.00 for the positive control sera.

Part II – long term consistency: Antibodies to ADV in the DBS were estimated by adapting the VP2 ELISA to DBS (Andersson et al., 2015). In brief, the DBS were eluted in PBS/T elution buffer (SVA, Uppsala), diluted to correspond to a final serum dilution of 1:800, and analyzed in micro titer plates coated with VP2 antigen diluted in PBS. The same batch of reagent solution was used for samples collected in October and in February. Positive and negative controls were employed at each micro titer plate, and the OD of each plate was adjusted to a value of OD_{450} = 1.00 for the positive control sera. The previously evaluated cut-off value of $OD_{450} \geq 0.15$ was considered as seropositive reaction for AD (Andersson et al., 2015). To further categorize the mink according to disease progression, the data were categorized using cut-off points corresponding to different levels of serum protein pathology according to previously made definitions (Cepica et al., 2012; Andersson et al., 2015). As seen in Figure 1, a value of $OD_{450} < 0.50$ corresponds to an A:γG ratio of >8 and was categorized as healthy, while a value of $OD_{50} \geq 0.83$ corresponds to an A:γG ratio of < 5 and was categorized as diseased.

Statistical analysis

In part I, data was normally distributed and statistical calculations comparing the consecutive sampling occasions were made with a two one-sided test for equivalence (TOST). Since any observed variance in ADV antibody levels could be a combination of the methodological variance and the physiological variance, an equivalence range was decided to correspond to ±0.1 OD based on previous studies of the coefficients of variation for the quantitative VP2 ELISA (Andersson et al., 2015). Equivalence was assessed by determining whether the 95% confidence interval for the observed difference between the two measurements was within this predefined range of equivalence.

In part II, data was not normally distributed but bimodal. Statistical calculations comparing the two sampling occasions were made with the non-parametric paired-samples sign test using the signtest command. The Spearman rank correlation between the two sampling occasions was also calculated. In order to evaluate whether the infection status as well as the disease progression of the individual mink were preserved from the first to the second sampling, the data were tabulated and visually assessed.

All statistical analyses and graphical presentations were made in Stata (StataCorp, College Station, Texas 77845, USA).

Results

Part I - Short term stability

In total, 120 samples were collected (60 each for the day-to-day and the week-to-week experiment, respectively) and complete records were obtained from 109 animals. Incomplete records were caused by broken tubes during centrifugation. The day-to-day analyses comprised 54 comparisons. Regarding week-to-week stability, 60 sera were complete on week one and two, and 55 sera were also available on week 3. The mean OD_{450} values are shown in Table 1 and 2 for the two groups of animals, respectively. The 95% confidence intervals for the comparisons of the different measurements were all within the predefined interval of equivalence (δ=±0.1 OD_{450}) and it was concluded that the measurements were equal.

Part II – Long term consistency

The median OD_{450} value was 0.58 (range: 0.04 – 1.5) in October. In February the year after, the median OD_{450} value had increased significantly (P = 0.001) to 0.72 (range: 0.03 – 2.1). The Spearman rank correlation between the two samplings was 0.53 and a scatter plot of the two samplings is presented in Figure 2.

Of the 350 mink that were analysed, 83% were categorized as seropositive (OD_{450} ≥ 0.15) during the October sampling and 90% during the sampling in February, respectively (Table 3). Approximately every second mink (55%) that was seronegative in October had seroconverted in February (32 out of 58 mink), while 3% of the mink that were seropositive in October were categorized as seronegative in February

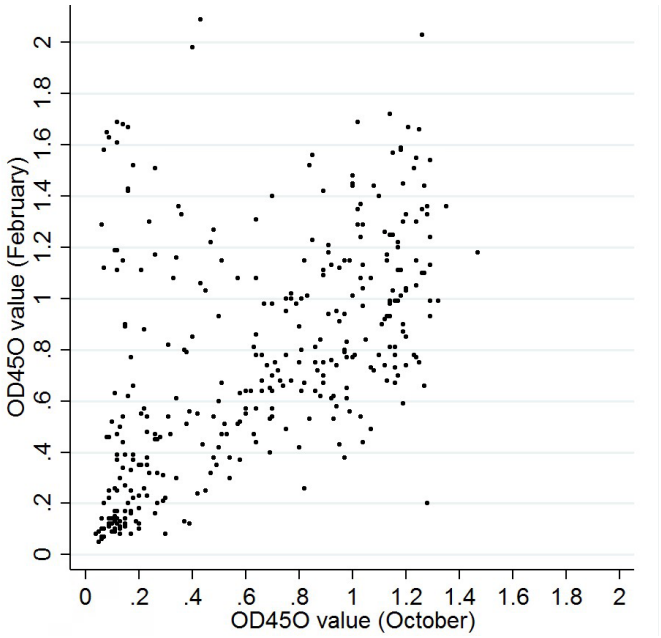

Figure 2: *Individual ADV ELISA OD_{450} values for 350 non-Aleutian, juvenile mink from one mink herd with a typical pattern and frequency of clinical symptoms of chronic Aleutian disease sampled repeatedly in October and February, respectively.*

Table 1: *Summarizing statistics and results from the two one-sided test for equivalence for the comparison between optical density values of blood samples from mink days 1 to 3 analyzed using a VP2 ELISA detecting antibodies against Aleutian mink disease virus in 54 mink from two herds*

Comparisons	(n)	Mean OD-value(SE)		Mean difference (range)	P-value*
		1st measurement	2nd measurement		
Day 1 vs Day 2	26	1.03 (0.08)	1.03 (0.08)	0.00 (-0.03 – 0.03)	< 0.01
Day 1 vs Day 3	28	0.86 (0.09)	0.82 (0.09)	0.04 (0.01 – 0.08)	< 0.01

** The greater of the two p-values of the two-sided equivalence test is shown in the Table. A P-value of <0.05 confirms no significant difference between the comparisons, i.e. rejects the null hypothesis that the absolute difference between the measurements is equal or greater to the predefined interval of equivalence of ±0.1*

Table 2: *Summarizing statistics and results from the two one-sided test for equivalence for the comparison between optical density values of blood samples from mink week 1 to 3 analyzed using a VP2 ELISA detecting antibodies against Aleutian mink disease virus in 60 mink from two herds*

Comparisons	(n)	Mean OD-value (SE)		Mean difference (range)	P-value*
		1st measurement	2nd measurement		
Week 1 vs Week 2	60	0.97 (0.04)	0.90 (0.04)	0.07 (0.04 – 0.09)	< 0.01
Week 2 vs Week 3	55	0.91 (0.04)	0.94 (0.04)	-0.03 (-0.06 – 0.00)	< 0.01
Week 1 vs Week 3	55	0.97 (0.04)	0.94 (0.04)	0.04 (0.01 – 0.07)	< 0.01

** The greater of the two p-values of the two-sided equivalence test is shown in the table. A P-value of <0.05 confirms no significant difference between the comparisons, i.e. rejects the null hypothesis that the absolute difference between the measurements is equal or greater to the predefined interval of equivalence of ±0.1*

(10 out of 292 mink). The mean OD_{450}-value ± SD (standard deviation) of these ten mink was 0.23 ± 0.09 in October, and all ten mink had an OD_{450} value in October which was slightly above the cut-off (range: 0.15- 0.39).

Table 3: *Distribution of mink categorized as Aleutian disease virus (ADV) seropositive or seronegative for 350 white juvenile mink from one mink herd sampled in October 2012 and February 2013 using OD_{450}=0.15 as a cut-off value for a positive ADV result*

		February	
October	**n**	**Seronegative**	**Seropositive**
Seronegative	58	26	32
Seropositive	292	10	282
Total	**350**	**36**	**314**

Of the 147 mink that were categorized as healthy (OD_{450}<0.50) during the October testing, 64% were categorized healthy in February as well, 23% as diseased and 13% as ambiguous (Figure 1 and Table 4). Of the 203 mink categorized as non-healthy (OD_{450}≥0.50) during the October testing, 92% were categorized non-healthy in February as well. The remaining 8% were categorized as healthy in February; the majority of these were originally categorized as ambiguous (Figure 1 and Table 4).

Table 4: *Number of healthy (OD_{450}: < 0.50), ambiguous (OD_{450}: 0.50 – 0.82) and diseased (OD_{450}: ≥0.83) mink determined by a VP2 Aleutian disease virus ELISA of 350 non-Aleutian, juvenile mink from one mink herd tested in October and in February the subsequent year*

	October	**Whereof in February**		
		Healthy	**Ambiguous**	**Diseased**
Healthy	147	94 (64%)	19 (13%)	34 (23%)
Ambiguous	66	12 (18%)	34 (52%)	20 (30%)
Diseased	137	5 (4%)	42 (31%)	90 (66%)
Total	**350**	**111**	**95**	**144**

Of the 137 mink that were categorized as diseased (OD_{450}≥0.83) during the October testing, 66% were categorized diseased in February as well, 31% were categorized as ambiguous while 4% were categorized as healthy (Figure 1 and Table 4). A majority of the mink (75%) that were categorized as non-diseased (the healthy and the ambiguous) during the October testing (n=213) were categorized the same in February. The remaining 25% had increased OD_{450} values and were categorized as diseased.

Discussion

When evaluated in the short term, i.e. between consecutive days as well as consecutive weeks, the estimated antibody levels were comparable and did not exceed the predefined equivalence interval based methodological variance for the VP2 ELISA previously published (Andersson et al., 2015). This shows that the VP2 ELISA is an excellent tool for quantitative estimation of the ADV antibody levels in mink in larger herds where the sampling for serological screening of many thousand mink typically extends over a couple of weeks.

The second part of the study was concerned with the long term consistency of the antibody level and the results showed a significantly higher mean antibody level to AD in February compared to in October. The most apparent reason for this general increase in antibody levels is disease progression, either in mink not yet infected in October or in infected mink experiencing a gradual increase of immunoglobulins typical of AD (Kenyon et al., 1963; An et al., 1978). Since the level of ADV antibodies are highly correlated to the level of gamma globulins (Farid and Segervall, 2014; Andersson et al., 2015), another possible explanation for the difference could be that the ADV levels were influenced by seasonal changes in gamma globulin concentrations in ADV infected mink (Bazeley, 1976; Farid and Segervall, 2014). However, the correlation between the two sampling occasions was poor, indicating that the antibody levels in the individual mink were not affected in a similar way by the above mentioned factors and that there may be other factors that affect the long term consistency in ADV antibody levels. For instance, decreased antibody levels between the two samplings could mirror "resistant" animals or animals with non-persistent infection (An and Ingram, 1978). Thus mink with low to decreasing concentrations of antibodies to ADV could thereby be of interest to select for breeding.

It could also be argued that the observed difference in antibody levels between October and February was caused by a methodological variance. However, the equivalence interval was defined as OD_{450}±0.1 based on the coefficient of variation during the previous validation of the VP2 ELISA (Andersson et al., 2015). In this study, the median absolute difference

Table 5: *Hypothetical calculation of the impact on the genetic base in a mink farm if culling ADV-positive females both in October and February employing different cut-offs in the ADV-ELISA. The results obtained in the present study has been extrapolated into a hypothetic farm with a base of 200 female adults that annually give birth to 500 female kits*

		Selection threshold		
		Seronegative (OD_{450} < 0.15)	Healthy (OD_{450} < 0.50)	Non-diseased (OD_{450} < 0.83)
Initial number of females (genetic base)	(n)	200	200	200
Females born	(n)	500	500	500
Total number of females	(n)	700	700	700
October				
Incidence of females above cut-off	(%)	83	58	39
Potentially culled due to high OD_{450}-value	(n)	581	406	273
Potentially remaining breeding females	(n)	119	294	427
February				
Incidence of females above cut-off	(%)	55	36	15
Potentially culled due to high OD_{450}-value	(n)	65	106	107
Potentially remaining breeding females	(n)	54	188	320
Adults required for the next season	(n)	200	200	200
Balance (basis for selection)	**(n)**	**-146**	**-12**	**120**

When using the ADV antibody level for grading animals based on their disease status, variations in the antibody level would consequently also lead to different estimations of the ADV progression in the breeding animals. Hence, the grading order of the animals, based on the estimated ADV antibody levels, would also vary between the October and February samplings. Within a control program, this means that the time point of sampling mink for making a selection within a control program will influence the animals selected for breeding. To address this problem, we investigated different categorization of ADV infection and AD progression based on ADV antibody levels, and how well the categorization was preserved between the two samplings (Table 5). By categorizing mink as either seropositive or seronegative in a screening program it could, based on our results, be expected that more than every second seronegative mink would seroconvert during winter. This would lead to a large culling percentage after the first sampling since many herds have a proportion of seropositive animals exceeding 80% (Andersson and Wallgren, 2013). In addition, culling approximately 50% after the second sampling would leave only a small percentage of the animals as a residual (8%; Table 5).

By categorizing mink based on the disease progression, an OD_{450} value of 0.50 or 0.83 could be used as the chosen cut-off for the classification of mink, and the number of females that could be spared for the next breeding season would increase in comparison to only keeping seronegative mink (Table 5). If selecting only healthy mink, *i.e.* employing OD_{450} < 0.50 as cut-off, our results indicate that approximately one third of the mink that were healthy in October could be expected to change category during winter (Table 4). The number of animals remaining after the second culling in February would then again be less than the number of mink needed in order to preserve the size of the breeding stock. If instead only mink that are diseased would be culled, *i.e.* use OD_{450}≥ 0.83 as cut-off, the number of animals that potentially could be spared for the next season would increase and leave freedom to select for other traits such as fur quality and litter size. Another advantage with a higher cut-off, at least at the first culling, is decreased risk of culling the animals that are AD tolerant, *i.e.* the mink which will go from a higher to a lower AD level or animals that will conserve a low antibody level and thereby escape disease progress. By employing a higher cut-off value and thereby keeping more mink, there will of course always be a balance act with an increased risk of viral spread from seropositive mink.

Of course, Table 5 should be looked upon as a hypothetical approximation based on a limited data set, and would have gained from data from more

herds. For instance, the herd scrutinized kept all tested mink from the October sampling regardless of their level of ADV-antibodies, which most likely increased the viral load and caused a higher prevalence of seropositive/non-healthy animals in February. However, even though the OD_{450} values can differ between herds, as well as within herds over time, it still leaves an idea about how the ELISA can be used as a tool for selection of breeders by altering the cut-off value based on the actual progress of the disease control program in the herd.

One important final remark is that several mink went either from being classified as ambiguous to healthy or from diseased to ambiguous, and this underlines the importance of being careful with the interpretation of the disease status of mink classified as ambiguous. To achieve a clearer view of the development of the ADV antibody levels over time, it would be interesting to follow the mink on a more frequent basis to document a more detailed pattern of the fluctuations of the antibody levels in a long term perspective. In addition, if the ELISA should be used as a predictor of breeding result in a control program for AD, the sampling time at which the ADV antibody level best can predict the breeding performance of the female needs to be more carefully evaluated before such a tool can be implemented.

Conclusions

ADV antibody levels in mink are comparable over a two-week period showing that the VP2 ELISA can be used for quantitatively estimating the ADV antibody level even when the screening extends over a couple of weeks. In contrast, antibody levels in samples collected four months apart differed significantly. If the ELISA should be used as a predictor of breeding result in a control program for AD, the optimal sampling time at which the ADV antibody level best can predict the breeding result of the female needs to be carefully evaluated.

Ethical Approval and Informed Consent

The study was approved by the Swedish Ethical Committee in Gothenburg (Dnr 117-2012), and the farmers participated voluntarily in the experiment. At all stages of the study, efforts were taken to minimize any pain, stress and discomfort of the animals.

Author's Contributions

Anna-Maria Andersson (AMA) and Per Wallgren (PW) conceived, designed and performed the experiments. Ann-Kristin Nyman (AN) and AMA analysed the data. All the authors contributed in manuscript writing and approval.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

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