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Distribution of Aleutian mink disease virus contamination in the environment of infected mink farms

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ABSTRACT

Control and eradication of Aleutian Mink Disease Virus (AMDV) are a major concern for fur-bearing animal production. Despite notably reducing disease prevalence, current control programs are unable to prevent the reinfection of farms, and environmental AMDV persistence seems to play a major role regarding this issue. In this study 114 samples from different areas and elements of seven infected mink farms were analyzed by qPCR in order to evaluate the environmental distribution of AMDV load. Samples were classified into nine categories, depending on the type of sample and degree of proximity to the animals, the main source of infection. Two different commercial DNA extraction kits were employed in parallel for all samples. qPCR analysis showed 69.3% positive samples with one kit and 81.6% with the other, and significant differences between the two DNA extraction methods were found regarding AMDV DNA recovery. Regarding sample categorization, all categories showed a high percentage of AMDV positive samples (31%–100%). Quantification of positive samples showed a decrease in AMDV load from animal barns to the periphery of the farm. In addition, those elements in direct contact with animals, the street clothes and vehicles of farm workers and personal protective equipment used for sampling showed a high viral load, and statistical analysis revealed significant differences in AMDV load between the first and last categories. These results indicate high environmental contamination of positive farms, which is helpful for future considerations about cleaning and disinfection procedures and biosecurity protocols.

1. Introduction

Aleutian disease (AD) or mink plasmacytosis is caused by the Aleutian mink disease virus (AMDV), a member of the family *Parvoviridae* recently classified as *Carnivore amdoparvovirus 1* (Canuti et al., 2015). In adult mink, AD causes a generalized systemic syndrome, including fur damage and reproductive disorders such as infertility, abortions and reduced litter size (Broll and Alexandersen, 1996), as well as a typical interstitial pneumonia with high mortality in mink kits (Alexandersen and Bloom, 1987). Consequently, the entry of AMDV to mink farms results in considerable economic losses for producers, and therefore this disease is considered the major concern for the mink fur production industry worldwide (Espregueira-Themudo et al., 2011; Farid et al., 2012).

Since there is currently no effective treatment or vaccine against AMDV, most fur producing countries have instituted control and surveillance programs aimed at limiting the economic impact of the disease for this sector. In general, control programs developed in most countries, including Spain, are based on serological testing of breeding animals by counterimmunoelectrophoresis (CIEP) or ELISA. When a farm shows a positive result, test-and-removal or stamping-out strategies are usually applied. Establishment of these control programs produces an early and significant reduction in the prevalence of the disease, which later slows down despite most farms maintaining test-and-removal strategies without interruption, mainly due to the re-infection of farms (Farid et al., 2012). In this regard, it has been demonstrated that the presence of AMDV infection on a farm in the preceding season, the farm size or the proximity to infected neighboring farms are risk factors for farm reinfection (Christensen et al., 2011; Espregueira-Themudo et al., 2012; Farid et al., 2012). Other suggested possibilities that could explain the failure of these control programs include diagnostic difficulties, the persistence of AMDV in the environment, transmission through infected wild fauna or even the virus strain (Farid et al., 2012).

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The contact between farms has been suggested to be the critical point in the maintenance of the infection and also an impediment to achieve the eradication of the disease in a region (Christensen et al., 2011). Transmission between farms could occur through contaminated materials, vehicles or personnel, and is also favored by the high resistance of AMDV to different physical and chemical treatments (Hahn et al., 1977). Regarding this, we previously confirmed the efficacy of a simple swab-sampling method combined with qPCR analysis to indicate the existence of AMDV DNA in dependencies, equipment and furniture of positive farms, differently from negative control farms where no AMDV DNA contamination was found (Prieto et al., 2014). Environmental testing has also been useful to identify the routes of transmission for other virus such as influenza both in human disease and swine production (Boone and Gerba, 2005; Neira et al., 2016). Therefore, a study of the contamination of different farm elements with AMDV would be helpful to evaluate the distribution of the virus in the environment of infected farms, as well as to identify contaminated fomites and possible routes of AMDV transmission. Nevertheless, it must be taken into account that DNA purification from environmental samples is usually difficult due to the existence of a huge variety of PCR inhibitors, so the DNA extraction method for this type of sample has to be chosen carefully (Schrader et al., 2012).

Thus, the main aim of this study was to evaluate the distribution and viral load of AMDV in infected mink farms by qPCR analysis of environmental samples, establishing different sample categories not only for different farm elements but also for visitors, workers and vehicles. In addition, two different commercial DNA extraction methods were evaluated for environmental samples.

2. Methods

2.1. Included farms, sampling method and sample categorization

Seven AMDV-infected farms were included in the study, six in northwestern Spain (Galicia) and the remaining one in the south of France. All of them were negative farms that became infected between 2011 and 2014, showing seroprevalence values between 16.56% and 68.82% prior to environmental sampling.

Environmental samples (n = 114) were collected between 2013 and 2015 and classified into nine categories based on the type of sample and the proximity to animals (Table 1). The sampling method consisted of swabbing each sampling area for 20–30 s with a dry sterile cotton swab (11 mm in diameter). Swab heads were then placed in sterile 12 ml screw-cap tubes by breaking the wooden stick of the swab. Samples from the category "Visitors" (coveralls and boot covers) were always taken at the end of the visit, which had a duration of 30 min. At the laboratory, the tubes were frozen at −20 °C until processed.

2.2. Sample preparation, DNA extraction and qPCR performance

Prior to DNA isolation, 5 ml of sterile phosphate-buffered saline with 0.05% Tween 20 were directly added to each sample tube and vortexed for 1 min (all reagents supplied by Sigma-Aldrich, Missouri, United States). After 15 min of settling, 1 ml of supernatant from each sample was taken and placed in a sterile Eppendorf tube, and then kept at −20 °C until DNA isolation was performed.

All samples were processed in parallel by two commercial DNA extraction kits: method A (Nucleospin® Soil, Macherey-Nagel GmbH & Co KG, Düren, Germany) and method B (High Pure PCR Template Preparation Kit, Roche Diagnostics GmbH, Mannheim, Germany). DNA was extracted from 200 µl of supernatant, following the manufacturers' instructions in both procedures (in the case of the method A, the lysis reagent SL1 was selected from the two available lysis reagents, and the optional reagent Enhancer SX was not used). The extracted DNA was collected in 100 ml of elution buffer and frozen at −20 °C until qPCR was performed.

For qPCR analysis, a commercial kit targeting the NS1 gene was employed (Genesig Advanced Real-Time PCR Detection Kit for Aleutian Disease Virus, PrimerDesign™ Ltd., Southampton, UK). Conditions for qPCR (thermal protocol, serial dilutions for standard curve) were the same as those described by Prieto et al. (2014). All qPCR reactions were run on an Applied Biosystems ABI Prism 7500 thermocycler (Thermo Fisher Scientific, Waltham, USA).

2.3. Data analysis

For both DNA extraction methods, number and percentage of AMDV positive samples, range and mean of AMDV copies and mean standard error for each category were determined from the qPCR results. Next, the number of AMDV copies of positive samples was 10-log transformed, and Welch's robust ANOVA test with Dunnett's T3 *post-hoc* analysis was conducted in order to determine the existence of significant differences among categories for each DNA extraction method. In addition, agreement between the results of methods A and B for positive/negative classification of all samples was assessed by Cohen's kappa and McNemar's test. Also, a Student's *t*-test for dependent groups was applied to positive samples for both protocols in order to study the differences in the mean of AMDV copies recovered for each method. All statistical analyses were performed with the IBM SPSS Statistics v.20 package (SPSS Inc., Chicago, IL, USA).

3. Results

Seventy-five out of 114 samples tested positive by both DNA extraction methods, while 4 out of 114 only showed positive results for method A and 18 out of 114 only for method B; thus, method B was able to detect more positive samples than method A (81.6% and 69.3% respectively). The initial number of NS1 copies was calculated by means of the obtained standard curve ($y = -3.318x + 37.207$; $R^2 = 0.999$; efficiency = 100.18%). The results of AMDV quantification for DNA extraction methods A and B are summarized in Table 2. As shown in Fig. 1, a higher number NS1 copies was observed for the first categories (in direct contact with or closer to animals) with respect to the last categories (no direct contact to animals). For method A, *post-hoc* analysis revealed significant differences between "Cages" with respect to "Street" ($p = 0.022$), "Effluents" ($p = 0.004$) and "Periphery" ($p = 0.007$), and between "Catching gloves" with respect to "Effluents" ($p = 0.026$) and "Periphery" ($p = 0.044$). On the other hand, method B showed the existence of differences between "Cages" with respect to "Effluents" ($p = 0.011$) and "Periphery" ($p = 0.016$), and between "Soil/Manure" with respect to "Effluents" ($p = 0.033$).

Regarding the different DNA extraction methods used, the agreement between both methods for classifying all samples as positive or negative showed a kappa value of 0.49, which corresponds with "moderate" agreement (Landis and Koch, 1977). In addition, McNemar's test found statistically significant differences between the classifications obtained by each method ($p = 0.004$). Finally, taking into

Table 1
Number and type of samples included in each sample category.

Category n°	Category name	n	Description
1	Cages	14	Cages and nest walls
2	Soil/Manure	9	Barn soil and manure under cages
3	Catching gloves	11	Gloves for animal immobilization
4	Slaughter box	8	Boxes for animal slaughter in the pelting season
5	Facilities	18	Warehouses and social dependencies
6	Visitors	11	Coveralls and boot covers
7	Street	12	Street clothes and footwear, farmers' vehicles
8	Effluents	16	Water outputs from the farms
9	Periphery	15	Silos and soil from farm entrance