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Diagnosing Aleutian mink disease infection by a new fully automated ELISA or by counter current immunoelectrophoresis: A comparison of sensitivity and specificity

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A B S T R A C T

Aleutian disease (AD) is a severe disease characterized by hypergammaglobulinemia causing multiple symptoms such as acute renal failure, arteritis, reduced reproductive performance and pneumonia in mink. AD is caused by the parvovirus Aleutian mink disease virus (ADV) and diagnosed primarily based on ADV serology sometimes supplemented by organ PCR analysis. In Denmark, approximately 3.5–4 million serum samples are tested every year for the presence of anti ADV antibodies as part of a national eradication program. The present study compares the diagnostic performance of the two most commonly used assays for serological screening for Aleutian disease: counter current immunoelectrophoresis (CIEP) and ELISA. In total, 3810 mink were sampled in doublets and analyzed by CIEP and a newly developed fully automated ELISA. The results show that the two assays have a comparable diagnostic performance with the ELISA having a higher sensitivity but lower specificity than the CIEP assay. The ELISA has been approved by the Danish authorities for diagnosing Aleutian disease in mink.

1. Introduction

Aleutian disease in mink (*Neovison vison*) is caused by the parvovirus Aleutian disease virus (ADV). In neonatal mink kits the disease can cause acute fatal pneumonia with virus replication in type II pneumocytes (Alexandersen, 1985; Alexandersen et al., 1988). In adult mink the disease is characterized by hypergammaglobulinemia which can result in immune-complex mediated glomerulonephritis, arteritis and reduced reproductive performance (Porter et al., 1969, 1973; Alexandersen et al., 1988). Aleutian disease is considered to be the disease causing the greatest financial loss in production of fur breeding animals worldwide. Therefore, the eradication of Aleutian disease is a major concern for the fur breeding community. So far, one of the most successful strategies has been national eradication programs based on screening and culling of the infected animals. The screening programs have traditionally been based on diagnosing ADV using counter current immunoelectrophoresis (CIEP). Increasingly, ELISA's are being developed to replace the CIEP analysis (Knuuttila et al., 2009). ELISA's have the advantage of being easily automated whereas the CIEP analysis is very labor intensive with many manual tasks and not suited for automation. The CIEP assay seems to be in good agreement with PCR analysis for the presence of ADV DNA in spleen and lymph nodes (Jensen et al., 2011).

ADV is a linear single stranded DNA virus with a 4.8 kb genome encoding three non-structural (NS1, NS2, and NS3), and two structural proteins (VP1 and VP2). The virus capsid consists of primarily VP2, which accounts for about 90% of the capsid proteins, and VP1, which is identical to VP2 with an additional 43 amino acids at the N terminus (Bloom et al., 1990, 1994, 1997). The ELISA developed by Knuuttila et al. (2009) uses recombinant VP2 as the antigen while the traditional CIEP uses whole ADV-G virus particles containing both VP1 and VP2 (Cho and Ingram, 1972; Uttenthal, 1992). The assay developed by Knuuttila et al. (2009) showed a good correlation with the traditional CIEP analysis, but was not in a format immediately appropriate for automation and screening purposes. At Kopenhagen Fur an ELISA antigen called ELISA Danad has been developed based on the whole ADV-G virus particles used in the Danad CIEP antigen. As both antigens are grown in the same way

Abbreviations: AD, Aleutian disease; ADV, Aleutian mink disease virus; CIEP, counter current immunoelectrophoresis; ELISA, enzyme linked immunosorbent assay; CRFK, Crandell Rees feline kidney; DBS, dried blood spot; CI, confidence interval; Sp, specificity; Se, sensitivity; RFU, relative fluorescence units.

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in CRFK cell lines and only the purification differs between them, they are presumed to display the same epitopes from both VP1 and VP2 on the surface. Previous studies have shown that mink develop antibodies against not only VP2 but also the VP1 specific protein sequence (Bloom et al., 1997). Therefore, the ELISA in the present study was developed using the ELISA Danad antigen containing whole ADV-G virus particles. The assay has been fully automated enabling the processing of 38,000 samples per day. It has been validated alongside the traditional CIEP analysis, thus, this study compares the validation results for both the CIEP and the automated ELISA.

2. Materials and methods

2.1. Samples

Samples from a total of 3810 mink were analyzed by CIEP and ELISA. Of these, 2436 were collected at 6 different ADV negative farms in Denmark and 1374 were collected at 6 different ADV positive farms in Denmark. The negative samples were presumed to be true negatives since all negative farms had been tested negative by CIEP annually for more than a decade. In addition these farms were located on the island of Zealand, which has not had an ADV outbreak since 1998 (unpublished data, Kopenhagen Fur). The positive farms were selected based on the prevalence of ADV as assessed in the routine CIEP screening program aiming for a range of prevalence from 5% to 90%. All positive farms were located in Jutland. Farms were selected to include both newly infected farms and farms continuously infected with ADV. The size of the farms varied from 1200 breeding females to 5200 breeding females. The samples were taken randomly around the farms. Blood was sampled by clipping a toenail and collecting the blood in doublets: Using an applicator and transferring it to a spot on a 12-spot dried blood spot card (DBS card) for the analysis by ELISA; and by collecting it in a heparinized capillary tube for CIEP analysis.

2.2. Statement on international standards for animal welfare

The blood samples used in this study were taken from animals kept under international standards for animal welfare. The blood samples were taken as part of the compulsory Aleutian mink disease virus state program in Denmark or as follow up of these compulsory blood samples. All blood was sampled in the same way and by experienced staff with minimal stress for the individual animal.

2.3. CIEP analysis

The CIEP analysis was performed as previously described (Cho and Ingram, 1972; Uttenthal, 1992) with minor modifications. Briefly, the capillary tubes were centrifuged and cut on a diamond cutter before the plasma was transferred to a well on an agarose gel. Danad antigen (Kopenhagen Fur, Glostrup, Denmark) was applied in the opposite well and the gel run for 30 min in Gelmann buffer (50.5 mM barbiturate, 40 mM Tris) at 4.5 V using a gel with opposing wells spaced 1 cm apart (corresponding to 4.5 V/cm). A positive result was indicated by the formation of a precipitate in the gel between the two wells. The results of the CIEP assay on samples from farm 2 to 6 were interpreted and recorded by two operators independently of each other.

2.4. Analysis by ELISA

An automated punching system cut a 3.5 mm disk from the center of each spot on the 12-spot DBS cards and transferred the blood sample on the disk to wells in an un-treated 384 well microtiterplate (NUNC, Roskilde, Denmark) referred to as “Extraction Plate”. A volume of 75 μL assay buffer (1% skim milk, 0.05% Tween20 in PBS) was dispensed in each well and the plate incubated for 5 h at room temperature. A black 384 well maxisorp™ microtiterplate (NUNC, Roskilde, Denmark) referred to as “Assay Plate” was coated by dispensing 6 μL ELISA Danad antigen (Kopenhagen Fur, Glostrup, Denmark) diluted 1:100 in PBS first and then adding 54 μL PBS in each well and incubated for 4 h at room temperature. After incubation the assay plate was emptied, blocked by addition of 115 μL assay buffer per well, and incubated for 1 h at room temperature. The assay plate was washed 3 times in wash buffer (0.05% Tween20 in PBS) and 40 μL assay buffer was transferred to each well. The content of the extraction plate was mixed by pipetting, 20 μL was transferred to the assay plate, and the content mixed by pipetting. The assay plate was incubated for 1 h at room temperature and washed three times in wash buffer. Then, 60 μL HRP labeled goat anti cat Fc specific antibody (Jackson ImmunoResearch, Newmarket, UK) diluted 1:3000 in assay buffer was added to each well in the assay plate, and the plate was incubated for 1 h at room temperature. The assay plate was washed three times and bound HRP visualized by addition of 60 μL of QuantaBlu working solution, prepared as described by the manufacturer (Thermo Fisher Scientific, Rockford, USA). The reaction was stopped after 5 min by addition of 50 μL QuantaBlu stop solution (Thermo Fisher Scientific, Rockford, USA). The assay plate was read at Ex 320 nm and Em 420 nm and the result reported in relative fluorescence units (RFU). The signal in RFU was normalized using Eq. (1) by subtracting the background signal from blank punches, dividing it by the signal from a positive standard also subtracted the background, and multiplying the result by 100. The resulting normalized value will be referred to as “Units” and abbreviated “U”.

Normalization of ELISA data using the signals in relative fluorescence units (RFU) of the sample, a blank sample, and a positive standard:

$$\text{Normalized value, Units} = 100 \times \frac{\text{RFU}_{\text{Sample}} - \text{RFU}_{\text{background}}}{\text{RFU}_{\text{Positive standard}} - \text{RFU}_{\text{background}}} \quad (1)$$

Table 1
Cross-classification of test results as positive (T+) or negative (T−) in a 2 × 2 table, according to the status of each individual animal tested by ELISA and CIEP.

	ELISA		Sum
	T+	T−	
CIEP			
T+	a	b	m_1
T−	c	d	m_0
Sum	n_1	n_0	n

2.5. Statistical analysis

All statistical analyses were performed in Excel 2010 or Openbugs (version 3.2.1 rev 781). The specificity of the two assays was assessed based on the samples from the farms presumed to be ADV negative. The specificity was calculated as shown in Eq. (2). The determination of the sensitivities of the two assays was performed as a comparison without gold standard as described by Dohoo et al. and Enøe et al. based on the assumption of independence between test results (Enøe et al., 2000; Dohoo et al., 2003). Data were sorted in a 2 × 2 table as shown in Table 1 and the sensitivities calculated as shown in Eqs. (3) and (4). The confidence intervals (CI) of the specificities were calculated using the exact binomial distribution (proc freq, SAS Inst.). Confidence intervals for sensitivities were