Journal of Virological Methods 171 (2011) 81–85

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Protocol

Implementation and validation of a sensitive PCR detection method in the eradication campaign against Aleutian mink disease virus

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Article history:
Received 23 June 2010
Received in revised form 3 October 2010
Accepted 7 October 2010
Available online 15 October 2010

Keywords:
Aleutian mink disease virus
AMDV
Mink
PCR
Counter-current immune electrophoresis

A B S T R A C T

Aleutian mink disease virus (AMDV) is a severe progressive disease causing multiple different clinical syndromes in mink. In Denmark, the disease is notifiable and under official control. The control programme, based on serological screening, has confined successfully AMDV to the northern part of Denmark. However, re-infections and new introductions of virus into farms require a confirmatory virological test to verify the positive test results of single animals and ultimately to investigate disease transmission. A one step PCR amplifying a 374-base fragment of the NS1 gene of AMDV was compared to the counter-current immune electrophoresis (CIE) routinely used in the serological screening programme. Mink organs ($n = 299$) obtained from 55 recently infected farms and 8 non-infected farms from 2008 to 2010 were tested by PCR, and the results were found to have a high correlation with the serological status of the mink. The relative diagnostic sensitivity of the PCR was 94.7%, and the relative diagnostic specificity was 97.9% when read in parallel with the CIE. PCR positive samples were sequenced and phylogenetic analysis revealed high similarity within the analysed AMDV strains and to AMDV strains described previously.

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1. Introduction

Aleutian mink disease virus (AMDV) has a severe impact on the health of mink worldwide, causing numerous clinical syndromes depending on virus strain and host factors such as genotype and age (Alexandersen et al., 1994b; Hadlow et al., 1983). Neonatal kits suffer an AMDV induced acute pneumonia (Alexandersen, 1986; Alexandersen et al., 1987, 1994a), whereas older mink can experience a chronic immune complex-mediated disease with hypergammaglobulinemia, glomerulonephritis (Alexandersen et al., 1988b; Porter et al., 1969), arteritis (Porter et al., 1973) reduced reproductive performance (Alexandersen, 1986; Broll and Alexandersen, 1996; Hansen and Lund, 1988) and/or non-suppurative meningoencephalitis (Dyer et al., 2000; Jahns et al., 2010). Furthermore, AMDV can infect other species such as skunk (Allender et al., 2008), ferret (Murakami et al., 2001; Saifuddin and Fox, 1996; Une et al., 2000), otter (Manas et al., 2001), raccoon (Oie et al., 1996) and Finn raccoon (Alexandersen et al., 1985).

AMDV is a single-stranded DNA virus of about 4.7 kb belonging to the genus *Parvovirus*, family *Parvoviridae* (Bloom et al., 1988b, 1990). The non-enveloped virion contains 3 non-structural proteins (NS1, NS2 and NS3) and two structural proteins (VP1 and VP2) (Alexandersen et al., 1988a; Bloom et al., 1988b; Qiu et al., 2006, 2007). The major NS1 gene together with the VP1 and VP2 genes are important for viral replication (Best et al., 2002, 2003; Bloom et al., 1988a; Christensen et al., 1993; Fox et al., 1999; Qiu et al., 2007). The VP genes encode for the majority of the viral capsid (Alexandersen et al., 1988a; Christensen et al., 1993) and together with the NS1 gene, the two VP genes are necessary for capsid assembly (Christensen et al., 1993). The VP2 protein harbours at least one epitope essential to the pathogenesis of AMDV including antibody binding, virus neutralisation and antibody dependent enhancement of infection in vitro (Bloom et al., 2001). Genetic variations between different AMDV strains have been described to occur at both the NS1 (Gottschalck et al., 1991, 1994) and VP2 gene (Bloom et al., 1988b; Schuierer et al., 1997).

A PCR amplifying a hyper variable region of the VP2 gene has been used to diagnose AMDV infection in raccoons and mink (Bloom et al., 1997a; Manas et al., 2001; Oie et al., 1996), ferrets (Murakami et al., 2001; Oie et al., 1996; Pennick et al., 2005; Saifuddin and Fox, 1996; Schuierer et al., 1997; Une et al., 2000) and a captive skunk (Allender et al., 2008). A semi-nested PCR targeting the NS1 gene has been used to characterise a high genetic diversity of AMDV strains from mink farms from Sweden (Olofsson et al., 1999) and Finland (Knuuttila et al., 2009b). Although Knuuttila et al. (2009b) compared the PCR results from different organs of 17

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doi:10.1016/j.jviromet.2010.10.004

mink with antibody detection-based serology, a larger statistical analysis has not compared so far AMDV PCR of organs with serology. Other studies compared AMDV serology with PCR on blood and found serology superior for screening because antibodies persisted beyond the detection of virus nucleic acids in blood, and intermittent detection of virus nucleic acids in blood was detected (Bloom et al., 1997b; Jackson et al., 1992, 1996).

The purpose of this study was to evaluate the diagnostic performance of the PCR in field material. A large number of mink organs ($n = 299$) was tested by a rapid and reliable one-step PCR, amplifying a 374 base (b) fragment of the NS1 gene. The PCR results were compared statistically to the serology results of the mink. Subsequent phylogenetic analysis of PCR positive samples obtained from November 2008 to December 2009 revealed high similarity to AMDV strains described previously (Christensen et al., in press).

2. Materials and methods

2.1. Mink tissue and cell culture samples

In total, 157 mink sampled from 63 mink farms were included in the study. Spleen and lymph nodes were sampled from mink originating from AMDV seropositive farms. Sera from these mink were tested by counter-current immune electrophoresis (CIE), slightly modified from Cho and Ingram (1972) and Uttenthal (1992). The CIE were performed by the Danish Fur Breeders' Laboratory, Glostrup, Denmark. The mink were sampled during the pelting season in November 2008 and 2009. AMDV is a notifiable disease in Denmark, and it is mandatory to test all farms annually using a sampling regimen reflecting the AMDV status of the farm and area in which it is located. In the case of AMDV-positive mink, it is recommended but not compulsory to stamp out the farm. The disinfection of the farm has to be approved by the veterinary authorities before restocking the farm. Accordingly, the animals in this study were infected recently; and with the exception of two farms, none of the farms have been infected for more than 6 months. Additionally, organs were collected from AMDV antibody-negative mink routinely submitted to the diagnostic service at the National Veterinary Institute, Aarhus, Denmark in 2009. In total, 109 CIE-positive and 48 CIE-negative mink were tested by PCR of spleens and 94 CIE-positive and all 48 CIE-negative mink were tested by PCR of lymph nodes (Table 1). From each mink, cardiac blood was collected at necropsy for CIE.

Spleen and mesenteric lymph nodes (0.1 g of each) were homogenised either manually or by the Qiagen Tissue Lyser (Qiagen, Hilden, Germany). The tissue was suspended in phosphate-buffered saline (PBS) containing 10% Qiagen protease (Qiagen) and incubated for 56 °C at 270 g for 1 h.

Crandell's feline kidney cells (CrFK) infected with AMDV-G (Danish Fur Breeders' Laboratory) were used for estimation of PCR sensitivity.

Table 1
Results of the mink samples tested by counter-current immunoelectrophoresis (CIE) and PCR on mink spleen and lymph nodes (lnn).

		CIE	
		Positive	Negative
PCR	Positive	99	1
spleen	Negative	10	47
PCR	Positive	74	1
lnn	Negative	20	47
PCR	Positive	89	1
parallel[a]	Negative	5	47

[a] Parallel – positive PCR result of either spleen or lymph node or both.

2.2. PCR

Total DNA was extracted from the mink tissue by QIAamp® Blood Mini kit according to manufacturer's instructions (Qiagen, Hilden, Germany). The DNA extraction procedure lasted 1 h. A positive spleen control from a Dutch outbreak and a negative spleen or lymph node control from a Danish AMDV free farm were included in each extraction. The positive and negative controls, respectively, were verified by PCR at the National Veterinary Institute at Lindholm. Corresponding serum samples were tested by CIE at the Danish Fur Breeders' Laboratory. A fragment of 374 b of the NS1 gene was amplified by PCR. The 100 μl amplification reaction mixture contained 5 μl sample DNA, 10 μl DyNAzyme™II DNA polymerase (Finnzymes, Espoo, Finland), 0.8 μl dNTPs (25 mM, Stratagene, Edinburgh, Scotland) and 10 pmol of each primer AMDV-F-7-H-PN1 (5′ CAT ATT CAC TGT TGC TTA GGT TA 3′) and AMDV-R-7-HPN2 (5′ CGT TCT TTG TTA GTT AGG TTG TC 3′) and DNAse free water. The PCR was initiated with 94 °C for 5 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s and a final elongation at 72 °C for 7 min. The PCR products were visualised by agarose gel electrophoresis, and from each mink farm, the sample resulting in the most distinct band on the gel was chosen for sequencing. DNA was purified for sequencing using the QIAquick PCR purification kit (Qiagen). Sequencing was performed with the PCR primers either by DNA Technology, Aarhus, Denmark or LGC Genomics, Berlin, Germany. The sequences were assembled and proofread by the use of CLC DNA Workbench (www.clcbio.dk). Sequence similarity was detected by BLAST search (blast.ncbi.nlm.nih.gov) and alignment with existing Danish AMDV sequences (Christensen et al., in press) by ClustalW (24) included in CLC DNA Workbench.

2.3. Titration of AMDV-G in Crandell feline kidney cells

CrFK cells (2×10^4 per well) were infected in 96 well microtiter plates with 100 ml of 5-fold dilutions of AMDV-G virus (Alexandersen, 1990) from undiluted sample and to a final dilution of 1:1 953 125 in PBS. The infected cells were incubated at 32 °C for 3 days at 5% CO_2 (Alexandersen, 1990; Bloom et al., 1980). For visualisation of infected cells, the cells were fixed in ice cold 96% ethanol, washed in 0.9% NaCl and incubated with polyclonal mink anti-AMDV sera diluted 1:200 in PBS with 0.1% Tween 20 (PBST). After 1 h of incubation at room temperature (rt), the cells were washed and incubated with protein A horseradish peroxidase conjugated (P8651, Sigma, Denmark) diluted 1:200 in PBST for 1 h at rt. The cells were washed again and stained with AEC according to manufacture's instructions (DAKO, Denmark). Infected cells were evaluated with an inverted microscope.

2.4. Estimation of PCR sensitivity

To estimate the sensitivity of the PCR 5-fold dilutions of AMDV, the infected CrFK cells were tested by the PCR. All dilutions started with undiluted sample and were diluted further 5-fold till 1:1 953 125 in PBS. Each sample was tested 4 times. The concentration of DNA was measured by NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA).

2.5. Statistical analysis

Diagnostic performance parameters (relative sensitivity, relative specificity, and Kappa with 95% confidence intervals (CI)) for PCR on spleen and lymph nodes, and parallel interpretation of PCR on spleen and lymph nodes (one or both tests positive are classified as positive) were calculated using the CIE as gold standard. Because the CIE is an antibody detection method and thus another diag-

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