Field evaluation of CIEP and PCR detection/removal control methods of Aleutian mink disease (AD) in Canada

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Abstract

Detection/removal control method of Aleutian disease by CIEP detection of ADV-antibody and farm or barn depopulation/repopulation, have been the recommended approaches of AD control since mid 1970s. The detection/removal was, at least under common N. American husbandry conditions, unsuccessful in controlling AD in a sustainable manner. Recently, attention was turned towards virus detection by PCR, and its use for AD eradication by removal of positive individual animals. In view of the common failures of CIEP to facilitate AD eradication, we were skeptical about premature acceptance of PCR-detection/removal, as the recommended control method. The frequent failures of CIEP test/removal were often blamed on breaches of bio-security. However, we believed that this method has been based on fundamentally wrong premise that the virus is primarily harbored by the infected animals. In reality, this sturdy parvovirus is harbored primarily in the contaminated environment through feces, saliva, urine, whelping, as well as through blood during bleeding for testing. While the use of both CIEP and PCR for monitoring of farms free of the virus remains certainly a valid approach, the data obtained in this study indicate that detection/removal by neither of the methods could facilitate real and lasting freedom from the virus, under the conditions of the study.

Keywords: Aleutian disease, control method, premise

Introduction

Control of AD has been a priority to mink industries all over the world since the appearance and spread of the disease in late 1040s and early 1950s (Gorham *et al.*, 1965). Hypergammaglobulinemia has been identified early as an important feature of the progressive form of the disease, and iodine agglutination test (IAT) has been the first 'laboratory test' to advance the diagnosis, albeit nonspecifically (Henson *et al.*, 1962). However, IAT identification of mink with AD related hypergammaglobulinemia, followed by culling, has led to very unsatisfactory degree of mitigation of AD related losses, since it only detects extreme cases of hypergammaglobulinemia, where gammaglobulin is at 0.2 g/1 ml, and albumin/gammaglobulin ratio is below 1 (Gorham *et al.*, 1965).

CIEP detection/removal AD control era started with the report of Cho and Greenfield (1978), on eradication of Aleutian disease of mink by eliminating positive counterimmunoelectrophoresis test reactors. In this report three farms were subjected to CIEP testing/cull. Tested took place over 4 years in November (pelting) and February (before breeding). Unqualified success was

reported on two farms, and on one there was one positive animal reported in each of the final two years. Undoubtedly, farmers and veterinarians alike noticed over the last more than 30 years of following this practice, that their experience with the program was generally disappointing. This recommended control method for AD has not generally been followed by lasting eradication, often in spite of the most rigorous biosecurity methods to prevent reintroduction (T. McLellean, J. Mullen, M. Bosco, personal communication). The inadequacy of this method is also indirectly acknowledged by recent interest in employment of the polymerase chain reaction (PCR) for the same purpose (Stahl *et al.*, 2010). This time, once again, a simplistic assumption was made. This assumption ignored the available knowledge regarding the long time presence of the virus in the hosts, the nature of virus shedding (Bloom *et al.,* 1994), and the resistant nature of the virus (Eterpi *et al.,* 2009; Sauerbrei and Wutzler, 2009). It was assumed that mere switch to PCR virus detection must accomplish what CIEP clearly did not accomplish through antibody detection. No wonder that this 'new hope' generated an excitement. In N. America, the main proponent was being invited to speak on his 'experience' with PCR to mink producer organization in the USA and Canada. The assumptions, once again, ignored the modes and frequency of virus shedding, as well as the resistant nature of the virus shed into the environment, encouraging premature and unfounded hopes. Such was the genuine conviction of M. Stahl (Pensylvania, USA), that he filed for patenting of his ideas regarding PCR and the AD control (Stahl *et al.,* 2010). Once again, on its surface, just like with CIEP, this approach of 'weeding out' the virus from mink operation seemed valid and supported by the voluminous body of literature attesting to specificity and sensitivity of PCR for virus detection. In order to save the Canadian mink industry expenditures and frustrations associated with premature implementation of a control method with unproven efficacy, we put in place a pilot study in which several aspects of ADV detection by PCR were tested. The primer selection for PCR and the procedure was developed and tested for specificity and sensitivity, the suitability of samples for the highest detection rate was investigated, several farms were tested by PCR for ADV incidence following the CIEP round of detection/cull, and finally five CIEP positive animals were followed for three months with respect to consistency of the PCR detection.

Materials and methods

Animals

For comparison of PCR detection effectiveness in blood, organ tissues (spleen and kidney), saliva, and rectal swabs, samples were collected on the same day from fifty 8 months old CIEP positive mink, which had been confirmed positive for over two months. All other mink were between six to twelve months of age when tested by PCR. These animals were previously CIEP tested negative during the round of CIEP testing when positive animals had been removed. The time elapsed between CIEP test/removal and rectal swab PCR testing is included with the results. Mink farms from Prince Edward Island and Nova Scotia, Canada, were included, that observed reasonable biosecurity precautions, including the wildlife proof fencing, and did not introduce animals from outside for a minimum of one year before the last CIEP testing that preceded the PCR testing.

Samples and sample processing

Blood was obtained by standard procedure of toenail cutting, followed by collection into glass capillary tubes. After centrifugation, the samples were shipped in cooled containers. Serum was collected on reception next day, and frozen at -75 °C until testing. We chose for monitoring of ADV presence after 'CIEP detection/cull' animals housed in common building and common area. The tests were perfomormed individually, or in some cases they were first pooled by groups of tens, with the intention that only the positive groups would be retested. This was based on the experimental determination that dilutions of samples 1:10 had no effect on the vast majority of positive samples.

Rectal and oral swabs saturated with collected and saliva, respectively, were made with sterile 'Cotton tipped applicator' (Puritan Medical Products Company, LLS, Guilford, Maine, U.S.) from farmed mink 7 to 12 months of age. Rectal swabs that had solid fecal material were discarded and taken again, as the DNA extracted from the fecal material often contained inhibitory substances for PCR. The swabs were individually placed in sterile plastic bags, shipped to the laboratory in a cooled container, and kept at 4 °C.

Counterimmuneelectrophoresis (CIEP). CIEP was performed using a commercial antigen (Antigen Laboratory of the Research Foundation of the Danish Fur Breeders' Association, Glostrup, Denmark) according to the manufacturer's instructions, in the Weymouth Aleutian Disease Laboratory, Weymouth, Nova Scotia, Canada.

DNA extractions

The samples were processed within 72 hours after collection. For testing of individual samples of rectal or saliva swabs, the swabs were immersed in 0.65 ml/swab of PBS and incubated at 4 °C for 24 hours. Then, each tube was wortexed and centrifuged at 12,000 rpm in a VWR microcentrifuge tube (VWR, IL USA) for 1 minute. Then 200 µl of the supernatant from processed rectal and oral swabs were recovered for DNA extraction from each of the individual swab sample. For group testing (groups of 10 samples), swabs were at first individually processed, and the recovered supernatants of ten animals (20 µl each) were pooled into 1.5 ml VWR microcentrifuge tube (VWR, IL USA).

Spleen, kidney, and blood, used for comparison of PCR ADV detection effectiveness for PCR were collected from 50 eight months old CIEP positive animals.

DNA extraction was performed according to the manufacturer's instructions by QIAGEN DNeasy Blood and Tissue kit for DNA extraction (Qiagen Inc., Gaithersburg, MD, USA). Final elution was done in 100 µl Elution Buffer.

Blood for PCR testing was collected into heparinized capillaries, and whole blood was subjected to DNA extraction. Spleen and kidney tissues were processed according to the manufacturer's instructions.

Polymerase chain reaction (PCR)

All nucleotide sequences of ADV that have been deposited to the Genebank were retrieved and aligned using CLC sequence viewer (http://www.clcbio.com) in order to design PCR primers in the conserved region of VP2 gene. PCR amplification was performed in a 10 µl reaction volume containing 1× PCR Buffer, 0.2 mM of each dNTP, 0.5 µM of each ADV F3444 (5'-GCAGAGATGAACAGARTTAGAC-3') and ADV R3831 (5'-CCTCATCTTCAAAGTGTGTG-3'), 0.25U of HotStarTaq Plus DNA polymerase (QIAGEN) and 2.5 µl of extracted DNA. The reactions were subjected to an initial incubation at 95 °C for 5 min followed by 40 cycles of 95 °C for 30 sec, 52 °C for 30 sec and 72 °C for 30 sec, and a final elongation at 72 °C for 10 min. Amplification products (10 µl) were separated using electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide and photographed under UV illumination.

Results

Comparison of PCR detection of ADV genome was performed on blood, saliva, rectal swabs, and pooled suspensions of spleen and kidney of 50 CIEP positive animals and 20 animals from ADV free facility (Table 1). The percentages of positive samples among blood, saliva, rectal swabs, and organ suspensions was 48%, 86%, 88%, and 86%, respectively. No positive samples were found among 20 animals from CIEP negative farm (see Figure 1 for an example of the test results).

Table 2 shows PCR results from rectal swab monitoring of animals from 5 farms, which the animals had been CIEP tested, and the positive animals were removed immediately after the testing. The number of PCR positive animals arising in farms where culling of CIEP positive animals took place range from 3.5% to 65%. Two rows that are highlighted represent group of

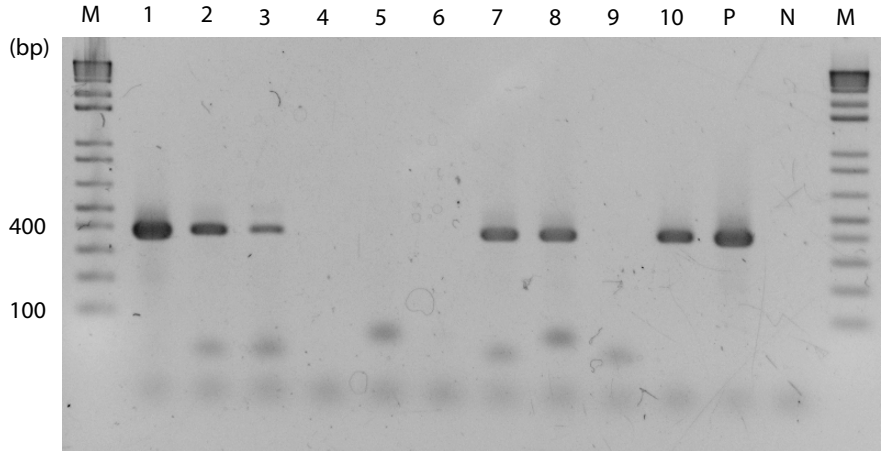

Figure 1. Example of results from group testing of pooled rectal swabs. Each group consist of 10 animals. Lane 1-3, 7, 8 and 10 were positive; P = ADV CDNA clone (positive control), N = DNA elution buffer used for extraction (negative control), M = 100 bp ladder.

Table 1. Simultaneous ADV PCR detection in blood, organ tissues (spleen and kidney), saliva, and rectal swabs in CIEP positive and negative animals.

Mink #	Blood	Saliva	Rectal swab	Spleen/ kidney	Mink #	Blood	Saliva	Rectal swab	Spleen/ kidney
1	-	+	+	+	26	+	+	+	+
2	-	-	-	+	27	+	+	+	-
3	-	-	+	-	28	+	+	+	+
4	-	-	-	-	29	+	+	+	+
5	-	-	+	-	30	-	+	+	+
6	-	-	-	+	31	-	+	+	+
7	-	-	+	-	32	+	+	+	+
8	-	+	+	+	33	+	+	+	+
9	-	+	-	+	34	+	+	+	+
10	-	-	-	-	35	+	+	+	+
11	+	+	+	+	36	+	+	+	+
12	-	+	+	+	37	+	+	+	+
13	+	+	+	+	38	-	+	+	+
14	-	+	+	+	39	-	+	+	+
15	-	+	+	+	40	-	+	+	+
16	-	+	+	+	41	+	+	+	+
17	-	+	+	+	42	+	+	+	+
18	-	+	+	+	43	-	+	+	+
19	-	+	+	+	44	+	-	+	+
20	-	+	+	+	45	-	-	+	-
21	-	+	+	-	46	-	+	+	+
22	-	+	+	+	47	+	+	+	+
23	+	+	+	+	48	+	+	+	+
24	+	+	+	+	49	-	+	+	+
25	+	+	+	+	50	-	+	+	+
51-70	-	-	-	-					

animals that were retested by PCR within close to two months of time. The first PCR test took place in early January, and the second in late February. Following the removal of 26% of animals on the first test, the second test identified 65% of PCR positive animals.

Table 3 shows PCR results on blood and rectal swabs of five CIEP positive animals, in three monthly intervals. The results shows the lack of consistent shedding, as both blood and rectal swab testing indicated intermittent shedding. Rectal swabs of three out of the five animals were positive at all three testing times. Two animals were rectal swab negative on one testing. As for blood, two animals were positive at all three testing times, two were positive at two times, and one animal was positive at one time.

Table 2. PCR monitoring of ADV incidence after CIEP detection/removal.

Farm	PCR test	Previous CIEP test and cull	PCR sample	Individual vs. group sample	No. of samples tested	No. of PCR pos. mink	% of PCR posit. mink
A	Dec	Nov	RS	I	50	23	46.00
A	*Jan*	*Nov*	*RS*	*I*	*187*	*48*	*25.67*
A	*Feb*	*Jan*	*RS*	*I*	*137*	*89*	*64.96*
B	Feb	Nov	RS	G	100	61	61.00
C	Feb	Nov	RS	G	75	36	48.00
C	Feb	Nov	RS	I	362	163	45.03
D	Mar	Nov	RS	G	51	11	21.57
D	Mar	Nov	RS	G	68	21	30.88
D	Mar	Nov	RS	I	210	41	19.52
E	Apr	Feb	RS	G	10	0	0.00
E	May	Feb	RS	G	105	35	33.33
E	Jun	Feb	RS	I	135	10	7.41
A	Jun	Feb	RS	G	26	11	42.31
A	Jun	Feb	RS	I	60	18	30.00
A	Jun	Feb	RS	G	17	4	23.53
A	Jun	Feb	RS	I	40	2	5.00
E	Aug	May	RS	I	58	2	3.45

Note: RS – rectal swab; I – individual animal sample; G – Group sample (n=10); Shaded and italicized area highlight two subsequent tests from the same farm/barn where PCR detection/removal was performed in early January, and the PCR testing took place in February.

Table 3. Results of the blood and rectal testing of CIEP positive mink, in 3 monthly intervals.

Animal I.D. #	October		November		December	
	Blood	Rectal swabs	Blood	Rectal swabs	Blood	Rectal swabs
1	+	+	-	+	+	+
2	-	+	-	-	+	+
3	+	+	+	+	-	+
4	+	+	+	+	+	+
5	+	-	-	+	+	+

Discussion

Regarding suitability of the investigated sample types (Table 1), the relative low percentage of positives among the blood samples was surprising. It could be argued that the use of heparin as an anticoagulant could be blamed for the poor performance of the blood samples, as there have been some reports suggesting that under certain conditions heparin may be inhibitory to PCR (García *et al.*, 2002). We do not believe, however, that this was the reason for the relatively poor performance of blood samples, compared to saliva, rectal swabs and organ suspension, as the DNA extraction kit used, was explicitly marketed for work with heparinized whole blood. We favor an explanation that the results reflected the true availability of ADV DNA in samples. More frequent shedding through feces and saliva may be related to sequestered local virus replication, and if true, this would constitute evolutionary advantage for the virus by increasing the chances of animal to animal spread. This would particularly be advantage to the virus in the natural habitat, since mink are in wild territorial animals. Apart from the unexpected higher detectability rate from rectal swabs (88%), compared to blood (48%), there were also other considerations that made us decide against the use of blood or saliva in farm monitoring. Perhaps the most important consideration was the potential of blood sampling to spread ADV and possibly other blood born pathogens. Droplets of blood splattering by the animals reacting to discomfort contaminate handlers' protective gloves and clothing which can not be disinfected or changed between individual animals. Furthermore, the stress associated with toenail clipping may be exacerbating stress induced conditions. Rectal swabs were collected with minimal stress to animals, and cross-contamination of rectal swabs can be better prevented, than cross-contamination of blood samples. While any contamination of toenail clippers would be very inefficient in antibody cross-contamination, it would very efficient in PCR contamination because of the extreme sensitivity of PCR virus detection.

Counter-immunoelectrophoresis (CIEP) gained wide use since the publication by Cho and Ingram (1972). The specificity of this test with respect to causative agent, was a major advance compared to the past iodine agglutination test (IAT), which detected a major consequence of progressive AD, hypergammaglobulinemia, albeit non-specifically. Serological detection of the virus brought great clarity into the process of determination whether ADV gained entry on a given ranch. Unfortunately, a great leap of thinking was made regarding the CIEP's potential to facilitate eradication of the virus, mainly because of the poor understanding of the virus and of the pathogenesis of the disease at that time. After the report of eradication with the use of CIEP 'detection/removal' (Cho and Greenfield, 1978), this method became promoted as a dogma in AD control. It can't be determined retroactively whether in those first reports true and lasting eradication did indeed occur, but we can say that over the period of 30 plus years of implementation of CEIP detection/removal the results were generally disappointing (T. McLellean, J. Mullen, and M. Bosco, personal communications). In our opinion, any appearance of eradication may have been fictitious, in that while temporarily there were no CIEP positive animals present, the virus remained to be present in some form on these 'eradicated' farms, only to resurface later when it gained access to animals. This could have been in the form of animals in the incubation period, false negatively testing animals, or this very resistant virus could have been present in the ground and later uncovered, or on the equipment, etc.

Throughout the period since introduction of CIEP detection/removal, admission of failure was associated with suspicions of errors in execution of this method of control, with plethora of areas were a farmer could have been blamed for unverifiable mistakes. For example, testing was not performed often enough, animals in the early stages of infection may have been missed, biosecurity on a farm may have been breached, or the virus may have been re-introduced, etc. Unfortunately, these speculations added nothing to improving the situation. Subsequently, not all farmers had the honesty to admit failures, particularly because of the economic and other implications. The testing did generate some sense of 'doing at least something', emanating from the belief that elimination of CIEP positive animals eliminates at least the bulk of the virus load. This belief may have been reasonable at early times when the properties of ADV parvovirus and the pathogenesis of the infections were not well known. The extreme resistance (Sauerbrei and Wutzler, 2009), the shedding, and the pathogenesis of ADV have begun to be better understood in 1980s and 19890s, CIEP detection/removal method of AD control should have been reviewed, but because of unavailability of alternative control method, it remained unquestioned. Elimination of the CIEP positive animals on at least two rounds, at the time of pelting and before breeding, especially if only small percentage of the animals tested CIEP positive, was sincerely but incorrectly believed to mean a negative status of the facility. Any future incidence was explained by errors in execution, timing of testing, or breaches of biosecurity.

Such dogmatic interpretation of reality of CIEP facilitated AD control was clearly incorrect, and it should have been realized long time ago. Massive shedding of this very resistant parvovirus (Uttenthal *et al.*, 1990; Gregersen and Roth, 2012) by feces, urine, saliva, as well as blood due to collection of blood for CIEP testing, may occur throughout the lifetime of the infected animals, even well before CIEP positivity develops (Hadlow and Kennedy, 1983). Under the common farming conditions in N. America, where excreta fall underneath the cages on the ground, and partly eaten, saliva contaminated food, become a lasting source of infection directly for other animals, and indirectly through personnel and fomites. Of course, in addition to these main modes of virus transfer within a facility containing infected animals, there are other highly probable means of spread of the virus, e.g. contacts during mating, contaminations through AD induced abortions and still births, handling of animals after weaning, movement of dust particles and/or aerosol droplets generated during power washing. Consequently, we believe, that 'CIEP detection/removal' method of ADV eradication has been based on faulty assumptions, and following the same assumptions, after switching the method of detection of infected animals from CIEP to PCR, would unlikely improve the outcomes of the 'detection/removal' control efforts.

In the past, the success rate of culling of CIEP positive animals, was monitored by CIEP again. Here, we monitored the rate of CIEP detectin/removal success by subsequent monitoring of the herds by PCR testing. Furthermore, in one facility, we followed the spread of the virus after culling of rectal swab PCR positive animals. Within less than two months, the incidence rate reached 65%, which can be partly explained by the failure to detect all infected animals, because of the intermittent shedding (Table 2). It could be argued that perhaps combination of CIEP and PCR testing/cull, could achieve better results. We do not believe that is likely, because of the previously stressed severe environmental contamination by this extremely resistant virus. We believe, that while depopulation and repopulation, following cleaning, disinfection, and facility

resting can be successful, but the threat of introduction will remain. At this time, we believe that 'on farm breeding for AD resistance' will be a reliable future method of control of AD.

While CIEP testing is highly satisfactory for monitoring of the CIEP status on ranches, it is our view that the recommendation of 'CIEP facilitated detection/removal eradication' should be discontinued as ineffective, and providing false security. It could be argued that 'CIEP detection/removal' is useful even if it does not accomplish eradication, by lowering the incidence of ADV infection on a farm. While this may be the case in a short term, we have documented rather rapid spread of ADV infections on several farms that practiced this method of presumed eradication.

Arguing against attainability of ADV eradiation from farm environment, we have shown that all on all ranches that we looked at, after a round of CIEP detection/removal, the incidence of ADV infection as monitored by PCR rapidly rose. This was rather unexpected, even though PCR can can identify both animals with advanced infection and animals in the early stages, as well as and animals in the incubation period. The high rates of PCR positivity can perhaps be also partly explained by difference of CIEP and PCR regarding their ability to react with the respective targets when they are a part of an immune complex. Immune complex is an important part of the pathogenesis of AD (Porter *at al.,* 1973). When immune complex is formed, only the excess of antibody in the blood is available to the CIEP test, and the complexed antibody is unavailable. Should all of the specific ADV antibody be complexed to the virus, the CIEP test would be negative. This is not the case for the PCR target, the viral DNA. DNA extraction procedure recovers viral DNA with equal effectiveness, whether it is complexed to antibody or not.

Setting aside the indisputably certain role of highly resistant AD parvovirus in the environment in difficulties with CIEP detection/removal, we examined in this work whether PCR on fecal swabs, blood, or saliva, had better potential as a tool for eradiation by 'detection/removal', than CIEP. While it might appear from the results presented (Table 2) that the rate of detection was very high attesting to a diagnostic power of the method, we do not know how this would compare with CIPE, if applied on samples taken at the same time. However, clearly, it is not reasonable to expect that PCR will identify every animal harboring replicating the virus, since the virus viral replication is restricted at the level of the individual cell and can be detected in only a small population of macrophages and follicular dendritic cells (Bloom *et al.*, 1984).

However, when applied under average farm conditions with reasonable biosecurity practices, in our hands neither CIEP nor PCR 'detection/removal' accomplished significant and lasting drop in incidence of the infection. Failures of CIEP are clear from the overall look at the PCR results performed after the CIEP detections/culls only few months prior to PCR testing (Table 2). In spite of the relatively short periods between CIEP and PCR, the percentages of PCR positive animals are quite high (Table 2). We discontinued the attempts to use PCR 'detection/removal' once we obtained the evidence of intermittent shedding in considerable number of animals (Table 3). Thus, in addition to heavy environmental contamination, another significant obstacle to eradication has been only intermittent presence of the virus in the samples (blood and rectal swabs). Thus in addition to the high cost associated with PCR testing, every round of testing leaves behind enough infected animals to maintain significant environmental contamination on the farms.

In conclusion, while either CIEP or PCR can be used to monitor appearance of ADV on farms previously free of the virus, application of either of the test to 'detection/removal eradication' program, at least under the conditions of this study, remained elusive. During the last 35 years, CIEP detection/removal was widely recommended in veterinary and farmers' literature, as effective. Frequent field failures have been blamed on presumed, but often unconfirmed and unprovable breaches of biosecurity. This situation led to difficulties with acquiring ADV free breeding stock, as Canada and USA, buyers must rely on unverifiable sellers' information regarding the ADV herd status. Often the economic incentive, combined with the lack of understanding lead to provision of supposedly ADV free breeding stock that in reality carries the virus into depopulated farms.

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