Canine Distemper Virus

Optimal age of vaccinating mink kits

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Introduction

In December 2008 the Canine Distemper Virus (CDV) was, after 16 years, reintroduced among the Dutch mink (*Mustela vision*) population. Clinical signs were first seen on non vaccinated farms and spread slowly to non-vaccinated neighbouring farms and farms that had received breeding stock originating from infected farms. The course of the disease was quite variable and much depending on the age (kits, females or males) at time of infection and vaccination status of the infected farm. Mortality rates varied between 2 and 40% and in December 2009 a total of 15 farms had dealt with a Distemper outbreak.

In the Netherlands, vaccination of mink kits against CDV was not common. It was estimated that approximately 25% of the breeding female population was vaccinated every winter. Despite starting to vaccinate all animals within the risk area (<10 kilometers) of affected farms, constantly new reports of Distemper outbreaks were made throughout 2009.

In reaction on the actual Distemper status, Dutch vaccination protocols are adapted to obtain good immunization at population level, both on and between mink farms.

Dutch mink population and farm management

During the past 10 years the Dutch mink sector has made big changes concerning farm sizes and Aleutian Disease (AD) status. The average size of mink farms is increased from approximately 3000 breeding females per farm in 2000 to an average of more than 4400 breeding females per farm in 2009. This great number of animals has several consequences concerning daily animal control, risk of infection and vaccination management.

Every year, when kits are born between late April and early May, total population increases at least 5 times. 95% of the newborn kits are born within a range of 3 weeks. Some kits will already be unprotected at time of vaccination; others will be too young to receive vaccination due to an incapable immune-system or the presence of maternal-derived immunity.

All leaflets of available mink vaccines tell that only AD free animals can be vaccinated which is infeasible for Dutch mink farmers. Today only 12 out of 200 farms in The Netherlands have had no AD positive animals over the past 3 years. Therefore AD has become an infection to be managed instead of being eradicated.

Main cause

Until today the main cause of 2008's Distemper outbreak is not known. One of the possibilities is import of already infected animals to a CDV naïve population of mink. Animals not showing clinical signs could have been infected already while being in their incubation period.
Wildlife reservoirs of CDV can also attribute to CDV outbreaks. 9 to 13% of Luxembourg's red fox population was positive to CDV antibodies (*Damien et al.* 2002). Similar results were found in a same survey conducted in Germany (*Frölich et al.* 2000).

Aims of the study

This study wants to point out how mink cope with Distemper vaccination performed on Dutch mink farms.

Pilot studies revealed that maternal antibodies of mink kits out of vaccinated females showed very low levels at the age of 6 weeks indicating that mink kits are thus less protected against CDV. The more than 4 week gap between low virus-neutralizing antibody levels and time of vaccination raises the question whether or not kits can be immunized before the age of 10 weeks. Because neutralizing antibodies against the Hemagluttinin protein (H) of CDV show correlation with protection against the disease, a Virus Neutralization (VN) test can be used as a marker for protection (*Norrby et al.* 1986).

The widespread presence of AD on Dutch mink farms and the leaflet of available vaccines indicating that only AD free animals can be vaccinated are reasons to investigate how, especially, AD infected animals react on Distemper vaccination.

With many speculations among mink farmers which Distemper vaccine induced better protection and because only two vaccines were available during the past CDV outbreak, both were used in this study.

Conducting the Virus Neutralization (VN) test is very labour-intensive. Therefore a more practical test is desirable. Results from a study with an Enzyme-Linked Immunosorbent Assay (ELISA) demonstrating IgG antibodies to Distemper in dogs show correlation with virus neutralizing antibodies found in the VN test (*Waner et al.* 1998). Other benefits of an ELISA assay are that results can be obtained much faster, its cheaper compared to the VN test and tests can be performed in less extended laboratory.

The aim of this study is to investigate the immune response of mink kits out of AD infected females, after Distemper vaccination at 6, 8 and 10 weeks of age, to determine the most optimal time of vaccination using a VN test. And to validate a commercial available CDV ELISA for the screening of mink sera by comparing the results with the results obtained from the VN test.

Virus Properties

Taxonomy

The Canine Distemper Virus (CDV) belongs to the family of Paramyxoviridae. This family is divided into two sub-families, the Paramyxovirinae and the Pneumovirinae. Pneumovirinae are morphologically different from Paramyxovirinae due to their narrower nucleocapsids. The Paramyxovirinae subfamily contains five genera, Avulavirus, Henipavirus, Respirovirus, Rubulavirus and Morbillivirus. The genus of Morbillivirus is the one where CDV belongs to. Other species of this genus are Measles Virus (MV), Cetacean Morbillivirus (CMV), Peste-des-petits-ruminants Virus (PPRV), Phocine Distemper Virus (PDV) and the Rinderpest Virus (RV).

This classification is based on the newest findings of the International Committee on Taxonomy of Viruses in 2009. Criteria were the latest known morphologic properties, genome organization, protein biologic activities and sequence relationships of encoded proteins (*Lamb et al.* 2001).
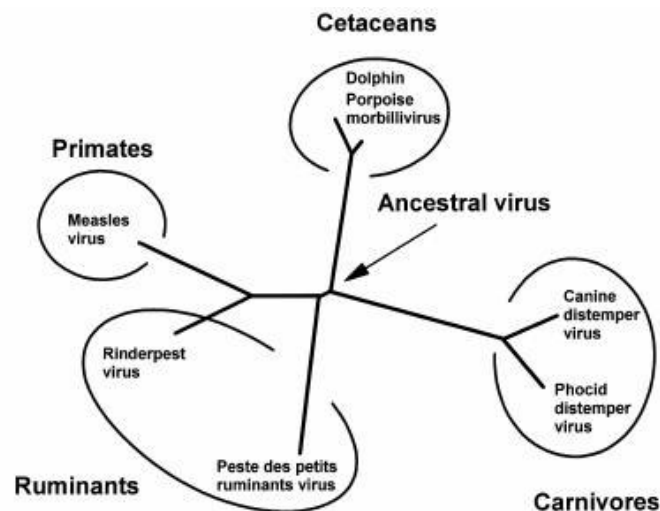

Figure 1. Phylogenetic tree Morbilliviruses(*Barrett* 1999).

Host range

Species within the Morbillivirus genus are well known viruses due to their impact on humans or animals with significant losses. MV with humans as its natural host, RV and PPRV had caused big outbreaks among cattle in history, PDV is closely related to CDV but is responsible for great losses among seals during different outbreaks in the past (*Müller et al.* 2008), CMV is the most recent specie added to this genus about 20 years ago as pathogen of cetaceans (*Bellière et al.* 2010) and finally CDV which has a broad range of natural hosts within the order of Carnivores (*Figure 1*) (*Beineke et al.* 2009).

Well-known species susceptible to CDV are dogs and foxes as species of the Canidae family, ferrets, mink and badgers of the Mustelidae family, raccoons and lesser panda of the Procyonidae family and skunk of the Mephitidae family. Other families with reports on Distemper outbreaks among their species are Hyaenidae, Ailuridae, Viverridae and Felidae (*Deem et al.* 2000).

Not only are seals susceptible to PDV, but they could also be affected by CDV while vice versa is also possible since Blixenkrone-Møller et al. reported CDV outbreaks in mink farms because of transmission from diseased seals (*Mamaev et al.* 1995, *Blixenkrone-Møller et al.* 1989, *Blixenkrone-Møller et al.* 1990).

Structure

All species within the Morbillivirus genus have a lipid envelope which encloses a helical nucleocapsid that contains a non-segmented negative sense RNA genome. When seen

trough an electron microscope all Paramyxoviruses show a typical herring-bone appearance (*Figure 2*) (*Barrett* 1999).
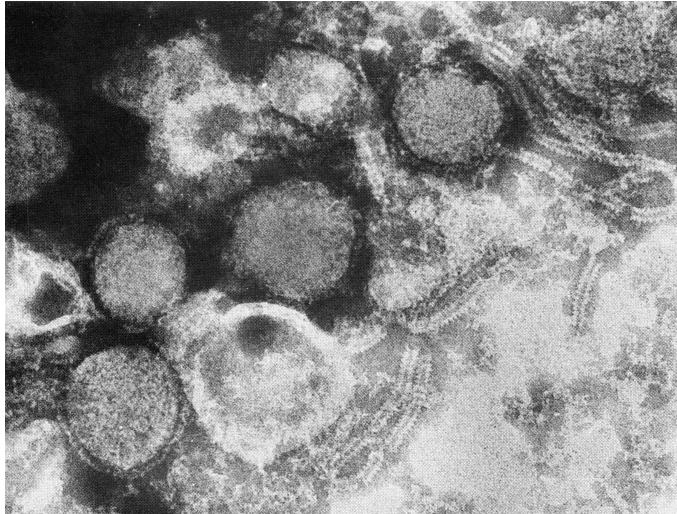

Figure 2. Electron Microscope picture of the Distemper virus (*Appel* 1987)*.*

The CDV genome is 15,690 nuclotides long and encodes for eight viral proteins located at six genes (*Figure 3*) (*Silin et al.* 2007).

The Nucleocapsid (N) is involved during virus replication: encapsidating the RNA genome into a nucleocapsid resistant against RNA-ase. During transcription it associates with the Phosphoprotein (P) and Large-Polymerase (L) and interacts with the Matrix protein (M) during virus assembly (*Lamb et al.* 2001).

The P gene is the only gene in the CDV genome with Open Reading Frames (ORF's) that encode for the P protein and two non-structural V and C proteins. The P protein is essential for viral RNA synthesis. The ORF responsible for transcription of the V protein lies in the middle of the P gene making the V protein responsible for a rapid viral replication in T-cells and inhibiting an interferon response. CDV strains lacking an expression of the C protein remain virulent and immunosuppressive, but its specific role is still unclear (*Lamb et al.* 2001, *Von Messling et al.* 2006, *Rothlisberger et al.* 2010).

The Matrix protein (M) has a central role in virus budding and virus assembly.

Two glycoproteins, the Hemagglutinin protein (H) and the Fusion protein (F) are both found as integral membrane proteins of host cells membranes. The H protein or attachment protein manages ion concentrations and pH inside the host cell and it makes virus particles attach to cells. Morbillivirus' H protein has in comparison to Respiro- or Rubulaviruses no neuramidase activity.

The F protein mediates pH-independent fusion of the viral envelope with membranes of host cells, making the virus capable of penetrating this membrane. After this reaction the nucleocapsid can enter the cytoplasm. The F protein expressed at membranes of infected cells can make these cells fuse with each other to form syncytia which can be seen as tissue necrosis or the so-called cytopathogenic effect (CPE).

The L protein, the last protein to be transcribed, has only 50 copies per virion. Together with the P protein it forms the viral RNA polymerase (*Lamb et al.* 2001).
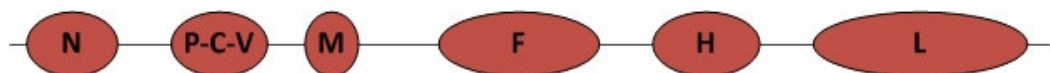

Figure 3. Schematic view of the proteins expressed from the CDV genome.

Pathogenesis, clinical signs and diagnosis

Animals suffering from Distemper can have no signs or show severe disease with mortality up to 90% in mink kits without maternal immunity. So the disease has a wide range of clinical manifestation. Incubation period in mink varies from 9 to 14 days in experimental infected animals and depends upon host's age and immune response, but also different virus strains can cause different clinical signs with variety in incubation period. Remarkable is the observation of higher mortality among pastel-type mink compared to wild-type mink during a Distemper outbreak suggesting genetically influence of dealing with the disease (*Beineke et al.* 2009, *Pearson et al.* 1987, *Appel et al.* 1995).

Pathogenesis

Virus is mainly spread airborne by saliva or other oro-nasal secretion, however, acutely infected animals can shed virus in all their body excretions. Susceptible animals can inhale virus particles or virus containing droplets. CDV cannot enter the body via the gastrointestinal tract or affect the fetus transplacentally (*Beineke et al.* 2009, *Pearson et al.* 1987).

First viral replication occurs in lymph nodes of the respiratory tract. After a local heavy replication of virus in macrophages and monocytes, CDV is spread by lymphatics and blood to other hematopoietic tissues including spleen, thymus, local lymph nodes, bone marrow, mucosa-associated lymphatic tissues (MALT) and macrophages in the lamina propria of the gastrointestinal tract. Several days after the first viremic phase, the second one follows with high fever and infections throughout the whole body (*Beineke et al.* 2009).

For protection against CDV both cellular and humoral immunity are important. Measurement of cellular immunity requires a lot more expertise and labour to be done. The humoral immunity can be measured with a virus neutralization test, demonstrating the virus neutralizing (VN) antibodies against the H and F proteins of CDV. These VN antibodies can be detected 6-10 days after vaccination with a titre peak between 14 to 21 days post vaccination and correlate with level of protection (*Rikula* 2008). A 1:20 VN antibody level after vaccination will protect animals against clinical disease (*Greene et al.* 2006).

Clinical signs

This first viremic phase leads to symptoms as swollen and watery eyes, an in the beginning serous and later purulent nasal discharge, making eyelids stick together and the nostrils occlude. With the eyes closed loss of appetite and anorexia occur and animals get dehydrated. A transient fever and lymphopenia occur 3 to 5 days post infection. Young kits will often die without developing signs of Distemper (*Beineke et al.* 2009, *Pearson et al.* 1987). One week after the appearance of the first symptoms, the feet can get three times larger as normal (*Pearson et al.* 1987). This type of clinical sign is less common in dogs where it is known as footpad hyperkeratosis or hard pad disease (*Koutinas et al.* 2004).

In a later stage diarrhea can occur. Animals can recover at this stage or die within a coma. Recovered animals, despite good appetite and normal appearance, will develop the so called neurotropic distemper. Neurologic signs prevail in this stage; animals frothing at the mouth, chewing meaningless or showing uncoordinated moves. These convulsions have a duration that varies from less than 1 hour to 2 days and mink mostly die after 1 or 2 convulsions (*Pearson et al.* 1987).

Diagnosis

A clear ante mortem diagnosis of Distemper is difficult to make. Animals recently vaccinated (< 3 weeks) will show the same IgM antibody levels as animals in the acute phase of an infection (*Appel et al.* 1995).

For mink, digital, nasal or eyelid-hyperkeratosis are clear indications for Distemper. Absolute lymphopenia, thrombocytopenia, regenerative anemia, decreased albumin and increased α- and γ-globulin concentrations are also indications for Distemper. Inclusion bodies can be detected in the cytoplasm of lymphocytes or in smears obtained from conjunctival discharge. However, there is a big chance of false negative results because these inclusion bodies are only present during the acute phase of infection (*Deem et al.* 2000).

Postmortem findings in mink suffering from Distemper are pneumonia, depletion of lymphopoietic organs and a widespread hyperkeratoris. Sometimes the only findings are

an enlarged spleen and atrophy of the thymus with all other viscera looking normal at macroscopic view. Sometimes lungs show consolidated foci or whole lobs (*Deem et al.* 2000, *Pearson et al.* 1987).

Histological evidence for Distemper is the presence of eosinophilic inclusion bodies in many organs such as the central nervous system (CNS), urinary bladder and bronchial epithelium. Also syncytial giant cells can be present in lungs or CNS's white matter. Lung, liver, lymph nodes, brain and spleen of suspected animals have to be collected for immunohistocytochemistry to provide definitive evidence for Distemper (*Deem et al.* 2000).

Vaccination history and possibilities

The first report of Distemper at mink farms was made in 1932 when Shaw described the disease in Canada. It caused great losses and the word Distemper was synonymous for fear among mink farmers.

In the same year the first application of an inactivated CDV vaccine was performed. This vaccine was prepared from inactivated brain tissue of mink with clinical signs of Distemper. More than 4000 mink were vaccinated on farms with an actual outbreak of CDV or which had a history of outbreaks in the past years. The morbidity on these farms decreased within a week after vaccination and also the daily mortality showed a sharp drop (*Shaw* 1932, *Pinkerton* 1940).

For more than 10 years this was the only possibility to temporarily protect mink against CDV; the vaccine couldn't develop an adequate immune response and animals vaccinated during winter were susceptible for CDV field strains in summer again (*Belcher* 1954).

The first use of an egg-adapted modified live vaccine for mink was done in 1951 during an outbreak of CDV at four mink farms. The virus was present at time of vaccination and further spread of the disease was prevented (*Hartsough et al.* 1953).

After that, many reports of cell-culture adapted CDV strains and immunization of susceptible animals were made. Both Haig, with his Onderstepoort strain obtained from chicken embryo's (*Haig* 1956) and Rockborn, with his Rockborn strain adapted to canine kidney cells (*Rockborn* 1960) started the development of several modified live vaccines for commercial use. With these vaccines the disease was well-controlled, but despite acquiring 100% immunity in sensitive dogs the Rockborn strain can give some post-vaccinational encephalitis in dogs. Haig's Onderstepoort strain has no reports of this postvaccinational encephalitis but there are indications that it has a lower seroconversion due to a different profile of the H glycoprotein in comparison to CDV field isolates (*Appel* 1999).

In the late 50s the application of a sprayable Distemper vaccine was tested. Under experimental circumstances the results were very encouraging, but when tested in the field the animals were much less protected. With a maximum of 68% protection of the vaccinated mink the results are not good enough for a commercial application (*Cabasso et al.* 1957, *Johnson et al.* 1957).

Nowadays the use of multiple combined vaccines is quiet popular, but the CDV-antibody response after vaccination is always poorer in comparison to immunize animals with the CDV-component alone (*Chappuis* 1995).

When dealing with a CDV outbreak, vaccination is an opportunity to consider. Only when clinical signs are limited to one spot on the farm vaccination is useful, otherwise vaccination will increase mortality and clinical signs become worse (*Hansen et al.* 1976).

Despite the acquired immunity after vaccination with modified live vaccines, there are some problems to deal with.

The present maternal immunity interfered with available modified live vaccines at time of vaccination. With a half-time of 9 days virus-specific antibody levels decrease when animals age and reach undetectable levels at the age of 12 weeks. Vaccination though is possible when kits reach the age of 10 weeks (*Welter et al.* 2000).

Another problem of the modified live vaccines is reversion to virulence when administered to wild-life species. Several reports were made from all kind of species that developed clinical signs after Distemper vaccination (*Montali et al.* 1983, *Ek-Kommonen et al.* 2003).

Nowadays a lot of research is done in the development of a DNA vaccine with adequate protection and without the negative side-effects of the modified live vaccines. Jensen et al. (2009) described DNA immunization of 5 days old mink kits with the H gene of a CDV vaccine strain. These animals received a total of four vaccinations and after the third immunization all kits showed virus neutralizing antibodies. When challenged with a virulent CDV strain these mink did not develop viraemia, lymphopenia or clinical disease (*Jensen et al.* 2009). These results are very encouraging for further investigation and DNA vaccination against CDV could be a serious possibility in the near future.

Aleutian Disease and immunity

Ranging from acute pneumonia in mink kits to mild non-progressive or fatal progressive disease in adult mink, Aleutian Disease (AD) has a broad scale of clinical manifestations (*Knuuttila et al.* 2009b).

The expression of AD present on mink farms depends on differences in virus-strains and mink-genotypes. Mink carrying the so-called Aleutian-gene are more susceptible to AD virus then others. Comparison between four virus-strains in non Aleutian genotype and Aleutian genotype mink ranged from fatal disease in the Aleutian genotype mink to no disease in the non Aleutian genotype mink (*Hadlow et al.* 1983).

AD is a non-enveloped, 4.7kb single-stranded DNA virus. As member of the Parvovirus genus it belongs to the family of Parvoviridae. The AD genome consists of 3 non-structural proteins (NS1, 2 & 3) and 2 structural proteins (VP1 & VP2). Most genetic variations are found in the NS1 and VP2 genes (*Jensen et al.* 2011).

Almost all newborn mink kits out of AD-negative females infected with high virulent strains of AD will develop an interstitial pneumonia and die most of the time. Adults and some other newborn kits will develop a chronic disorder characterized by plasmacytosis and immune-complex disease. Adult mink have a more rapid development of anti-AD antibodies and therefore less chance to develop acute disease (interstitial pneumonia). The factor whether or not this pneumonia will occur in newborn mink kits depends on the passive transfer of maternal anti-AD antibodies that will prevent acute disease, though chronic disease still can develop (*Alexandersen et al.* 1989).

However the exact pathogenesis of AD is not known and AD infected mink kits not always show clinical signs of disease, it is likely though that they have a comprised immune-system and their response to vaccination is uncertain.

Monitoring

Breeding stock is tested for AD prior to mating-time every year and positive animals are removed. Available tests are the low specific Iodine Agglutination test (iodine test) and high specific counter current electrophoresis (counter test). The counter test is useful for mink farms dealing with no AD history to screen farms on positive animals in an early stage. Iodine testing mainly concerns farms with moderate amounts of AD positive animals and detects animals with high levels of AD-antibodies. Although both tests have proven their application in the field, both have some strong disadvantages in comparison to more recent techniques available for serology.
The iodine test's main disadvantage emerges on farms with little number of positive animals but too many for the use of the counter test, or farms with so many positive animals leaving no animals behind for the next breeding season. The same problem emerges concerning the counter test; farms with too many positive animals are not being able to reduce their number of positive AD animals or eliminate the virus from their farm.

Both tests have proven their application in the past, but many Dutch mink farmers cannot take their AD management to a higher level. Although ELISA and Polymerase Chain Reaction (PCR) have been widely used in other sectors, they have no big implementation in the mink sector. Recent studies show encouraging results for implementation in serological eradication or management of AD on mink farms with help of both these techniques (*Jensen et al.* 2011, *Knuuttila et al.* 2009a).

Materials and Methods

Animals

Two Aleutian Diseased (AD) mink farms, infected for more than five years and stable at iodine testing (<10%) or ELISA AD testing prior to mating time (December-February), were selected. Past years different kinds of orientation studies were performed on the same two farms. In February 2010 approximately 25 females (Wild-type), not mated before, were selected on both farms as study animals. All animals were housed individually, had no signs of clinical AD or Distemper and were kept under normal farm conditions according to the guidelines of the Dutch Fur Association (NFE).

From April 28th to May 9th 2010 (week 0) selected kits were born. During week 1 kits were counted and litters with less than 5 kits or born outside the range of week 0 were excluded from the experimental group. Finally in week 6 the experimental group was formed out of litters with 5 or more healthy, grown well kits.

On both farms the study group consists of 15 females with their offspring. Three different vaccination strategies were performed dividing all experimental animals equally and randomly over the A, B or C group (5 females with ≥5 kits per group). All kits received vaccination with Febrivac 3-PLUS® or Biocom-P® in week 6. Both these vaccines contain the Clostridium Botulinum type C toxoid, an inactivated mink enteritis virus and the inactivated Pseudomonas aeruginosa serotype 5,6 and 7/8 components. Distemper vaccination for Group A was administered in week 6, for Group B in week 8 and Group C in week 10 with either Distemink® or Febrivac-DIST® equally distributed among all groups. These Distemper vaccines are freeze-dried and consist of a modified live CDV (*Table 1*).

Farm 1

Group	Date of birth	3-way Vaccine		Distemper Vaccine	
		Biocom-P®	Feb-3+®	Distemink®	Feb-Dist®
1A1	3rd May	Week 6		Week 6	
1A2	8th May		Week 6		Week 6
1A3	7th May	Week 6		Week 6	
1A4	30th April		Week 6		Week 6
1A5	1st May	Week 6		Week 6	
1B1	1st May		Week 6		Week 8
1B2	3rd May	Week 6		Week 8	
1B3	3rd May		Week 6		Week 8
1B4	30th April	Week 6		Week 8	
1B5	1st May		Week 6		Week 8
1C1	4th May	Week 6		Week 10	
1C2	28th April		Week 6		Week 10
1C3	8th May	Week 6		Week 10	
1C4	29th April		Week 6		Week 10
1C5	30th April	Week 6		Week 10	

Farm 2

Group	Date of birth	3-way Vaccine		Distemper Vaccine	
		Biocom-P®	Feb-3+®	Distemink®	Feb-Dist®
2A1	30th April		Week 6		Week 6
2A2	2nd May	Week 6		Week 6	
2A3	2nd May		Week 6		Week 6
2A4	2nd May	Week 6		Week 6	
2A5	7th May		Week 6		Week 6 + Week 10*
2B1	9th May	Week 6		Week 8	
2B2	5th May		Week 6		Week 8
2B3	30th April	Week 6		Week 8	
2B4	3rd May		Week 6		Week 8
2B5	28th April	Week 6		Week 8	
2C1	1st May		Week 6		Week 10
2C2	7th May	Week 6		Week 10	
2C3	2nd May		Week 6		Week 10
2C4	9th May	Week 6		Week 10	
2C5	28th April		Week 6		Week 10

Table 1 . Overview of experimental groups present on both farms with different vaccination times & products. First digit of the group represents Farm 1 (1) or Farm 2 (2), the next letters represents the experimental group (A, B or C) and the last digit indicates the cage number of each group (1 till 5). *Group 2A5 received a booster CDV vaccination in week 10

Vaccine administering and blood collecting

Animals were vaccinated according to the information leaflet: 1 ml of the 3-way vaccine administered in week 6 injected subcutaneously (s.c.) into the left hind leg of the animal. Freeze-dried Distemper vaccines were mixed before use and injected s.c. into the right hind leg in week 6, 8 or 10 depending on which group the animals belonged to.

Blood samples of all kits were gathered in week 6, 8, 10 and 14. Animals were fixated in catching gloves and peripheral blood samples (±3ml) were drawn by puncture of one of the *Venae jugulares* with serum tubes and 21G needles. In week 14 it was sometimes not possible to fixate the animals well for vein puncture so these animals were sedated with 0.1ml Zoletil® administered intramuscular (i.m.) in one of the hind leg muscles. The collected samples were stored in cooling boxes at the farms. After all samples were obtained they were centrifuged for 3 minutes at 6000 RPM and serum was divided in two 1.5ml tubes (one for the VN test and one for the ELISA assay). These 1.5ml tubes were frozen and stored at -20°C until assayed.

For Farm 2, blood samples of the females were collected at week 6 simultaneously with the kits. Samples of Farm 1's females were lost during the storage period.

Sometimes blood samples couldn't be obtained due to failure of vein puncture (Farm 1 n=4, Farm 2 n=8) or an animal died with unknown cause during the study period (Farm 1, n=1 after week 10).

Cell culture

Vero cells, kidney cells derived from the African green monkey, were grown in 75cm^2 Tissue Culture (TC) flasks with 15ml Dulbeco's modified Eagle medium (DMEM) containing 10% heat inactivated fetal calf serum and 5000 units penicillin and streptomycin. After washing the Vero cells with 10ml Dulbeco's Phosphate Buffered Saline without Mg and Ca (PBS0) the cells were trypsinized with 4ml Tyrpsin and single cells were dissolved in a medium appropriate for the dilution of the next passage.

Virus stock

Vero cells were grown as a monolayer in three 225mm^2 TC flasks with 80 percent of confluent cells. The CDV^{Ond} virus strain with a $10^{4.5}$ TCID$_{50}$/ml titer and adapted to Vero cells, was dissolved in 30ml DMEM containing 2% heat inactivated fetal calf serum, 5000 units penicillin, 5000 units streptomycin and 25 mg Gentamycin. Each flask was washed with 10ml of Dulbeco's Phosphate Buffered Saline containing 50mg/liter Diethylaminoethyl-Dextran (PBS DEAE), infected with 10 ml of the virus-containing solution and incubated for one hour at 37°C in a 5% CO_2 incubator. At the end of the incubation period DMEM 2% medium was removed, flasks were washed with 10ml Dulbeco's Phosphate Buffered Saline, with Mg and Ca (PBS Mg+Ca) and replaced by 25ml DMEM 2%. After six days of incubation at 37°C in 5% CO2 all flasks showed enough cytopathogenic effect (CPE) to harvest the new virus stock and flasks were emptied into two sterile 50ml tubes. These were centrifuged for 10 minutes at 3000RPM and transferred into one TC 75mm^2 flask leaving the cell pellet behind. Everything was divided into 1ml parts in 1.5 ml tubes and stored at -80°C as CDV^{Ond}.

Virus titration

100µl of DMEM 10% containing 1×10^5 cells/ml were added into a 96-wells plate and incubated for 1 day at 37°C in 5% CO_2. The next day after thawing, 200µl of CDV^{Ond} X+2 was dissolved in 800µl DMEM 2% and 5-fold dilutions were made. The 96-wells plate with the cells was emptied and washed with 100µl PBS DEAE per well, 100µl of each virus/medium mixture was added to each well except 2 wells for cell control. The 96-wells plate was incubated for 6 days at 37°C in 5% CO_2. After 6 days the CPE was scored and titer was determined as the highest virus dilution showing 50 – 100% CPE. As control the 96-wells plate was colored with 100µl of 0.75% Crystal Violet (CV) for 10 minutes. The CDV^{Ond} had a titer of $10^{4.38}$ TCID$_{50}$/ml and could be 12 times diluted to get a 100 TCID$_{50}$/50µl.

Serum neutralization

Serum samples were heat inactivated for 30 minutes at 56°C. Two-fold serum dilutions were made in micronic tubes starting with 80µl serum in 320µl DMEM 2% (1:5 till 1:640). CDV^{Ond} was diluted and 200µl of this solution is added to each dilution tubes and incubated for 1 hour at 37°C. 96-wells plates incubated with Vero cells were emptied and each well was washed with 100µl PBS DEAE. After washing, three 2-fold dilutions with 100µl of the serum/virus/medium mixture were added to the 96-wells plate. The plates were incubated at 37°C and CPE was scored microscopically after 6 days.
End titers were marked when 2 or more wells of the highest serum dilutions showed less then 50% CPE.
For every 5 96-wells plates (= 20 samples) a control plate was added. This control plate contained a negative control serum provided by N. Schuurman, Utrecht University, a positive control serum obtained from a 5 year old dog vaccinated by the latest vaccination guidelines from the Royal Dutch Veterinary Medicine Association (KNMvD), a back titration of the virus with ten-fold dilution steps, 100, 10 and 1 TCID$_{50}$/50µl, and cell control wells with only 100µl DMEM 2% per well.

ELISA

Three CDV-ELISA kits were supplied by European Veterinary Laboratorium (EVL) in Woerden. ELISA procedure was performed as described in the leaflet. Sera were screened as 3-fold dilutions in duplicates, a 1:30 till 1:810 dilution. Negative and positive control samples were provided for every test kit. Samples of more than 0.500 Optical Density (O.D.) read at 450nm were classified as positive.

Results

Neutralization assay

Each vaccinated group (A, B or C) consisted of 5 different litters at both farms. To analyze all these data, neutralizing titers were expressed as its reciprocal. Titers lower then 5 were recorded as 1, titers higher or equal to 640 were recorded as 640. These values were in turn transformed to their log_{10} reciprocal to simplify statistical analysis.

Neutralizing antibodies present at 6 weeks were significant lower (P<0.0001) on Farm 1 (*0.48 ± 0.07 N=75*) compared to Farm 2 (*1.09 ± 0.09 N=72*) (*Figure 4*).
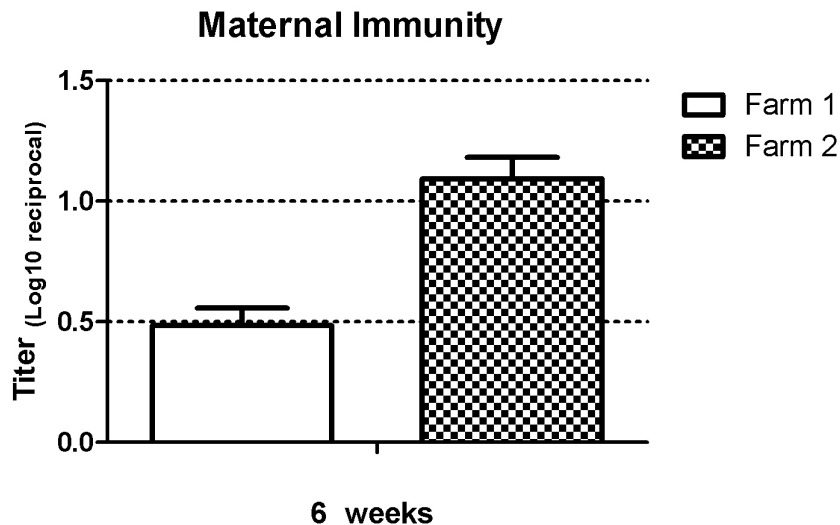
Maternal Immunity

Figure 4*. Maternal immunity present on both farms in 6 weeks. The error bar indicates the Standard Error of the Mean (SEM).*

During the experimental period a total of 295 serum samples were collected at Farm 1 and 292 at Farm 2. As shown in table 2, serum samples gathered in week 6 demonstrate that animals from Farm 1 (60%) had twice the animals with undetectable levels of neutralizing antibodies compared to Farm 2 (29%). In week 14 this percentage reversed to more than twice the percentage of animals with undetectable antibody levels at Farm 2 (31%) compared to Farm 1 (13%). The same trend was seen at Farm 2; however, the percentage of animals with undetectable levels of neutralizing antibodies present in week 6 was lower in all three groups. At 14 weeks of age the animals from Farm 2 also had a higher percentage of animals with neutralizing antibody levels less than 20 compared to Farm 1.

Group	N	% <5	% <20	
1A6	25	56	76	
1B6	25	64	76	
1C6	25	60	80	
Total Farm 1 6 weeks	75	60	77	
2A6	23	22	26	
2B6	24	21	21	
2C6	25	44	60	
Total Farm 2 6 weeks	72	29	36	
Group	N	% <5	% <20	% >80
1A14	24	29	29	33
1B14	23	9	13	57
1C14	24	0	0	89
Total Farm 1 14 weeks	71	13	14	62
2A14	18*	50	61	17
2B14	25	44	48	8
2C14	25	4	8	76
Total Farm 2 14 weeks	68	31	37	37

Table 2 Percentage of animals with <5, <20 or > 80 virus neutralizing antibodies in week 6 and 14 on both farms.
*Less animals because one cage received booster vaccination in week 10. These samples (n=5) are excluded from further statistical analysis.
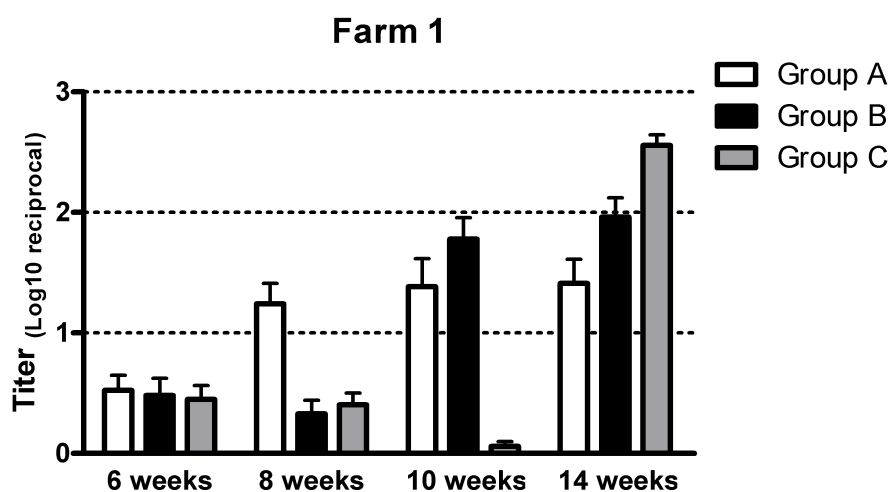
Figure 5. *Mean log$_{10}$ reciprocal of neutralizing antibodies with its Standard Error of the Mean (SEM) over the whole experimental period of Farm 1. Group A received Distemper vaccination in week 6, Group B in week 8 and Group C in week 10.*

Neutralizing antibodies found at 6 weeks in Group 1A (*0.52 ± 0.12 N=25*), showed a significant increase (P=0.0013) when measured two weeks after vaccination in week 8 (*1.24 ± 0.17 N=25*). The same results were found for Group 1B (P<0.0001) and Group 1C (P<0.0001) comparing week 8 and 10 and week 10 and 14 respectively (*Figure 5*).
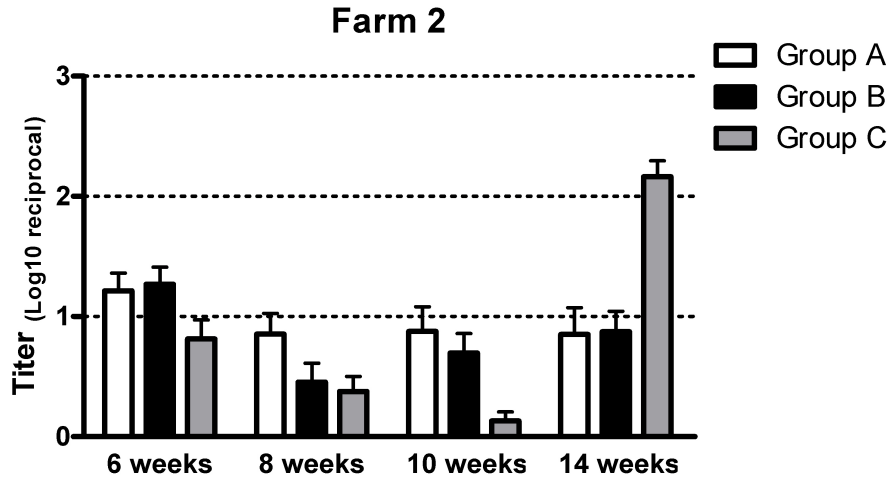
Farm 2

Figure 6. *Mean log$_{10}$ reciprocal of neutralizing antibodies with its SEM over the whole experimental period of Farm 1. Group A received Distemper vaccination in week 6, Group B in week 8 and Group C in week 10.*

Group A from Farm 2 showed a different trend in antibody levels compared to the A Group of Farm 1. The higher maternal immunity in kits at Farm 2 probably interfered with vaccination applied in week 6 (*1.12 ± 0.18 N=18*), showing no increase but a not significant (P=0.1196) decrease in mean neutralizing antibody levels in week 8 (*0.98 ± 0.19 N=19*). The little increase of neutralizing antibody levels in Group B after vaccination was not enough to make it significant. Group 2C though showed a significant increase of neutralizing antibodies comparing week 10 (*0.13 ± 0.07 N=25*) to week 14 (*2.16 ± 0.13 N=25*) with P<0.0001 (*Figure 6*).

At 6 weeks only Group 2B (*1.27 ± 0.14 N=24*) had significant higher levels of neutralizing antibodies compared to Group 2C (*0.81 ± 0.16 N=25*) with P=0.0380. Mean neutralizing antibody levels of Group 2A (0.85 ± 0.22 N=18) and Group 2B (*0.87 ± 0.17 N=25*) were significant lower compared to Group 2C (2.16 ± 0.13 N=25) at 14 weeks with P<0.0001 (*Table 3*).

Farm 1			Farm 2		
Groups Compared	*P value*	*Significant*	*Groups Compared*	*P value*	*Significant*
1A6 vs. 1A8	0.0013	Yes	2A6 vs. 2A8	0.5892	No
1B8 vs. 1B10	<0.0001	Yes	2B8 vs. 2B10	0.2879	No
1C10 vs. 1C14	<0.0001	Yes	2C10 vs. 2C14	<0.0001	Yes

Table 3. *P values extracted from two-tailed T-test for different experimental groups. First digit stands for Farm 1 (1) or Farm 2 (2). The next letter represents the experimental Group (A, B or C) and the last digit shows from which week (6, 8, 10 or 14) the results were obtained.*

Vaccination of Group 1A induced a significant increase of mean neutralizing antibody levels as said before. The significant higher level of neutralizing antibodies at time of vaccination in Group 2A (16) probably interfered with vaccination, causing a decline in mean neutralizing antibody levels after two weeks (7). Although the decline occurred, antibody levels remained stable over the whole study period.

Most remarkable findings come with the B Group, vaccinated in week 8. It was notable that Group 2B had no increased mean neutralizing antibody levels after vaccination unlike Group 1B and even Group 1A, both with similar antibody levels at time of vaccination. Group 1A and 1B did show significant increases after vaccination though, but vaccination of Group 2B in week 8 induced no significant increase of mean antibody levels despite a significant decrease of maternally derived antibodies between 6 (19) and 8 (3) weeks.

Group	6 weeks	8 weeks	10 weeks	14 weeks
1A	3	17	24	26
1B	3	2	60	91
1C	3	3	1	359
Group	6 weeks	8 weeks	10 weeks	14 weeks
2A	16	7	8	7
2B	19	3	5	7
2C	7	2	1	146

Table 4. Mean antibody levels for each group on both farms over the whole experimental time period.

Neutralizing antibodies of Farm 2's females were also assayed (*Figure 7*). Only one female had no detectable neutralizing antibodies while 7 out of 15 females had titers higher than 80. Neutralizing antibody titers were not significantly different between groups.
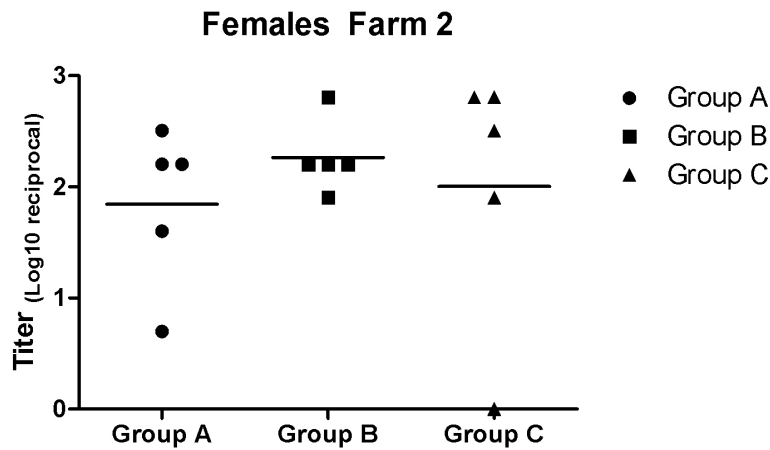
Figure 7. Neutralizing antibody titers from the females of Farm 2 for all three groups.

One cage with five kits belonging to Group 2A received Distemper vaccination at 6 weeks and was given a booster vaccination when the kits had the age of 10 weeks. The initial vaccination administered at 6 weeks (*1.54 ± 0.18 N=5*) showed no increase but rather a significant (P=0.0033) decrease of neutralizing antibodies in week 8 (*0.25 ± 0.25 N=4*). However, the booster vaccination showed no significant (P=0.0870) increase in mean neutralizing antibody titers comparing week 10 (*0.58 ± 0.36 N=5*) to week 14 (*1.70 ± 0.45 N=5*), 4 out of 5 kits had titers above 20 in week 14 compared to 1 out of 4 after the first vaccination (*Figure 8*).
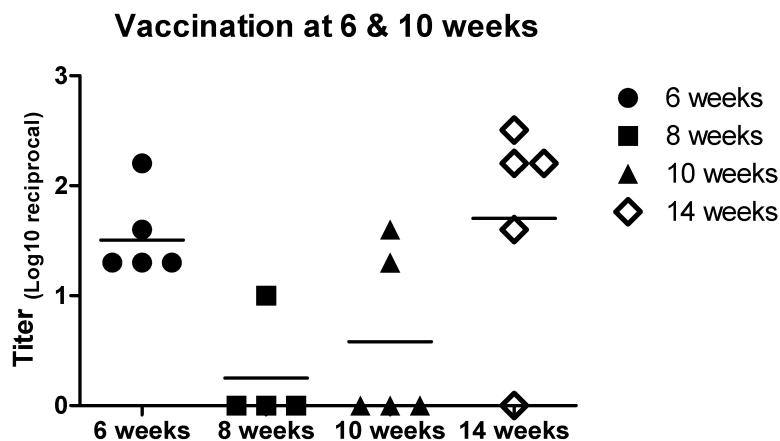

Figure 8. Effect of booster vaccination in Group 2A with the error bar showing the geometric mean.

For detecting differences in potency of both Distemper vaccines, samples from week 14 were divided into two major groups; the DISTEMINK® and the Febrivac DIST® group (*Figure 9*). Mean $\log_{10}$ reciprocal antibody levels from week 14 were compared. The DISTEMINK® group (*mean $\log_{10}$ reciprocal ± SEM, 1.47 ± 0.12 N=75*) had a significant lower mean antibody level compared to the Febrivac DIST® group (*1.89 ± 0.11 N=64*) with P=0.0127. Within the DISTEMINK® group 33 out of 75 samples (44%) had antibody levels equal or lower than 20. Concerning the Febrivac DIST® group this was only 13 out of 64 (20%). The DISTEMINK® group had 44% of all samples equal or higher than 80 compared to 72% of the Febrivac DIST® group (*Table 5*).
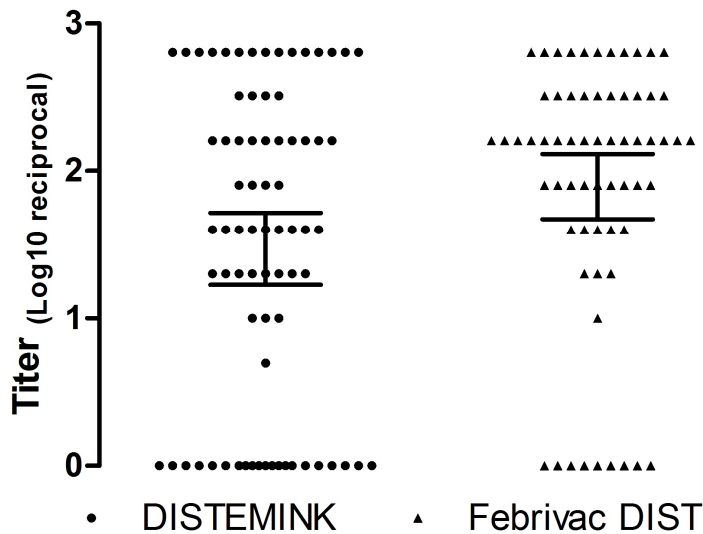
Figure 9. *Neutralizing antibody levels in week 14 divided into two vaccine groups; DISTEMINK® and Febrivac DIST®. Each dot represents one sample with the error bars showing the geometric mean and its 95% coincidence interval.*

Vaccine	Mean antibody level	% <20	% >80
Distemink®	30	44	44
Febrivac DIST®	78	20	72

Table 5. *Mean antibody levels, percentage lower than 20 and higher than 80 for both vaccine groups.*

ELISA assay

Results of the ELISA assay were compared with results obtained from the VN test. A total of 54 samples were compared and statistical analysis was performed (*Table 6*).
Although 16 samples (30%) had undetectable antibody levels in the ELISA compared to antibody levels of 80 or higher in the VN test, a correlation ($r^2=0.14$) was found between the CDV ELISA and the VN test with P=0.0049. Levels of neutralizing antibodies <5 found in the VN test are most of the time (11 out of 12) scored as undetectable in the ELISA test too.

Sample no.	ELISA	VN	Sample no.	ELISA	VN	Sample no.	ELISA	VN
1	1	1	10	30	80	19	1	1
2	1	80	11	30	1	20	1	320
3	1	1	12	30	160	21	1	640
4	1	80	13	30	320	22	90	640
5	1	160	14	1	160	23	1	1
6	1	80	15	30	160	24	1	1
7	1	1	16	30	640	25	1	1
8	30	160	17	30	640	26	1	1
9	30	320	18	1	20	27	1	10
Sample no.	ELISA	VN	Sample no.	ELISA	VN	Sample no.	ELISA	VN
28	1	10	37	1	160	46	1	1
29	1	20	38	90	80	47	1	1
30	1	20	39	1	160	48	30	10
31	1	20	40	1	320	49	30	20
32	1	20	41	1	640	50	80	90
33	1	80	42	1	640	51	30	160
34	1	40	43	1	640	52	30	640
35	1	160	44	30	640	53	90	640
36	1	160	45	1	1	54	90	640

Table 6. ELISA and VN test results compared. 1 indicates antibody levels <5.

Discussion

This study demonstrates that mink kits born out of AD infected females show adequate immune response after Distemper vaccination at the age of 10 weeks. On both farms Group C had significant increases of mean neutralizing antibody levels after vaccination. With 0% of the animals demonstrating undetectable levels of antibodies on Farm 1 and only 4% on Farm 2 these results are very acceptable under field conditions. The question whether or not these animals are protected against the diseases remains unanswered because not only the humoral immune response is involved in defeating the virus. Although Norrby et al. (1986) found correlation with neutralizing antibodies and protection against the disease, the role of cellular immunity is important too (*Nielsen et al.* 2009).

The assumption that mink kits have very low levels of neutralizing antibodies before the age of 10 weeks is confirmed. Mean neutralizing antibody levels in week 6 varied from 3 to 12 on Farm 1 and 2 respectively, and are both beneath the minimum level of protection found in dogs by Greene et al (2006).
With the minimum age of 10 weeks at time of vaccination according to the leaflet, many animals are already unprotected for at least four weeks. A Distemper virus circulating at this time will have a major impact, causing high rates of mortality among mink kits.

This problem can be solved by vaccinating animals at a younger age. With vaccination administered at the age of 6 and 8 weeks, neutralizing antibody levels showed different trends on two study farms.

On Farm 1 vaccination at the age of 6 and 8 weeks induced significant increases in mean antibody levels. Despite this significant increase, 29% of the animals in Group A had antibody levels lower than 20, making a large proportion of the animals still susceptible to a virulent CDV strain. Group B, however, had 13% of its animals with antibody levels beneath the suggested level of protection after vaccination. This percentage is quiet acceptable under field conditions and shows that protective levels can already be induced after vaccination at an earlier age.

The serum samples obtained from Farm 2 showed no increase in mean antibody levels after vaccination in week 6 and 8. Explanations for these results are not clear. The reason that vaccination of Group 2A failed can be dedicated to the high levels of maternal antibodies that interfered with the modified live virus of the vaccine. Group 2B had comparable levels of maternal antibodies in week 6, but at time of vaccination in week 8, its levels were equal to Group 1A and 1B where mean antibody levels significantly increased after vaccination.
Maybe the vaccine was not prepared or stored well and thereby lost its efficacy, but with no significant differences between both vaccines, this theory is less plausible.
Other possibilities are host specific factors. The higher levels of maternal immunity on Farm 2 present at 6 weeks could still have interfered with vaccination in week 8 despite its decrease compared to week 6; the exact levels of maternal antibodies that will not interfere with vaccination and thereby making the vaccination successful are not known.
Since the AD virus is circulating on both farms, this could also be a factor that makes the Distemper vaccination, and others, succeed or not. The exact pathogenesis of AD is still not known, but its impact on the animal's immune system is obvious. AD infected animals probably have a less functional immune system that could fail in up taking antigens or have lower immune responses after vaccination. However vaccination did not induce the desired antibody levels in Group 2B, CDV vaccination was successful in Group C though. To analyze the exact way in which AD interacts with the immune response to vaccinations, further research is needed.

Although mean antibody levels increased in Group 1A and 1B, there was still a proportion of animals that showed no immune response after vaccination. This proportion of susceptible animals declines when the animals age and reach acceptable levels on both farms after 10 weeks as prescribed by the leaflet. Due to differences between the experimental farms, vaccination of animals younger than 10 weeks can not blindly preceded without screening for antibodies before and after vaccination.

For each farm that will vaccinate its animals before 10 weeks of age, it is necessary to gain an insight into the CDV antibody status of mink kits at the age of 6 weeks. Together with the serological results and the risk in a certain extent of CDV outbreaks in the neighborhood or on the farm it can be decided to vaccinate animals earlier than 10 weeks.

To minimize the risk of low levels of antibodies after initial vaccination before 10 weeks a booster vaccination can be administered. Results of the booster vaccination applied in one cage did not show a significant increase four weeks after the second vaccination, but finally 4 out of 5 animals had antibody levels above the suggested minimum level of protection compared to 2 out of 5 before the last vaccination. In view of the large number of animals, with variable immune status and age, to be vaccinated at the same time, a booster vaccination could give better results.

First vaccination applied when the animals have the age of 6 weeks for example, will protect mink kits that already have unprotectable levels of antibodies and animals skipped or failed to induce an adequate immune response after the first vaccination have a second chance. This vaccination strategy is applied in dogs, but concerning mink, double costs and labour for two vaccinations will not have a lot of support among mink farmers.

The significant difference between both vaccines is remarkable because they only show this difference when divided into two major groups with no regard to which experimental group the samples belonged to. Comparing the vaccines was not a major aim of this study and to answer the question which vaccine induces better protection after vaccination, clinical trials are needed.

But with the overall look on the question whether or not vaccinating mink kits at a younger age and with the risk of interfering with maternal immunity, it is desirable to use a vaccine that has the best potency in inducing high antibody levels.

Although a correlation was found between the VN test and the ELISA assay its practical use can be questioned. With 30% of the samples showing no detectable antibody levels in the ELISA while more than 80 in the VN test, the interpretation of negative results tested with the ELISA assay is very hard and makes the test not useful in practice. With only the VN test as liable test method in determining antibody levels against CDV there will be no practical implementation because it is very labour-intensive. Because the importance to analyze the Distemper status in young mink kits before and after vaccination, a practical test is very desirable and an ELISA appropriate for the use of mink sera should be developed in a new study.

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Figures and Tables