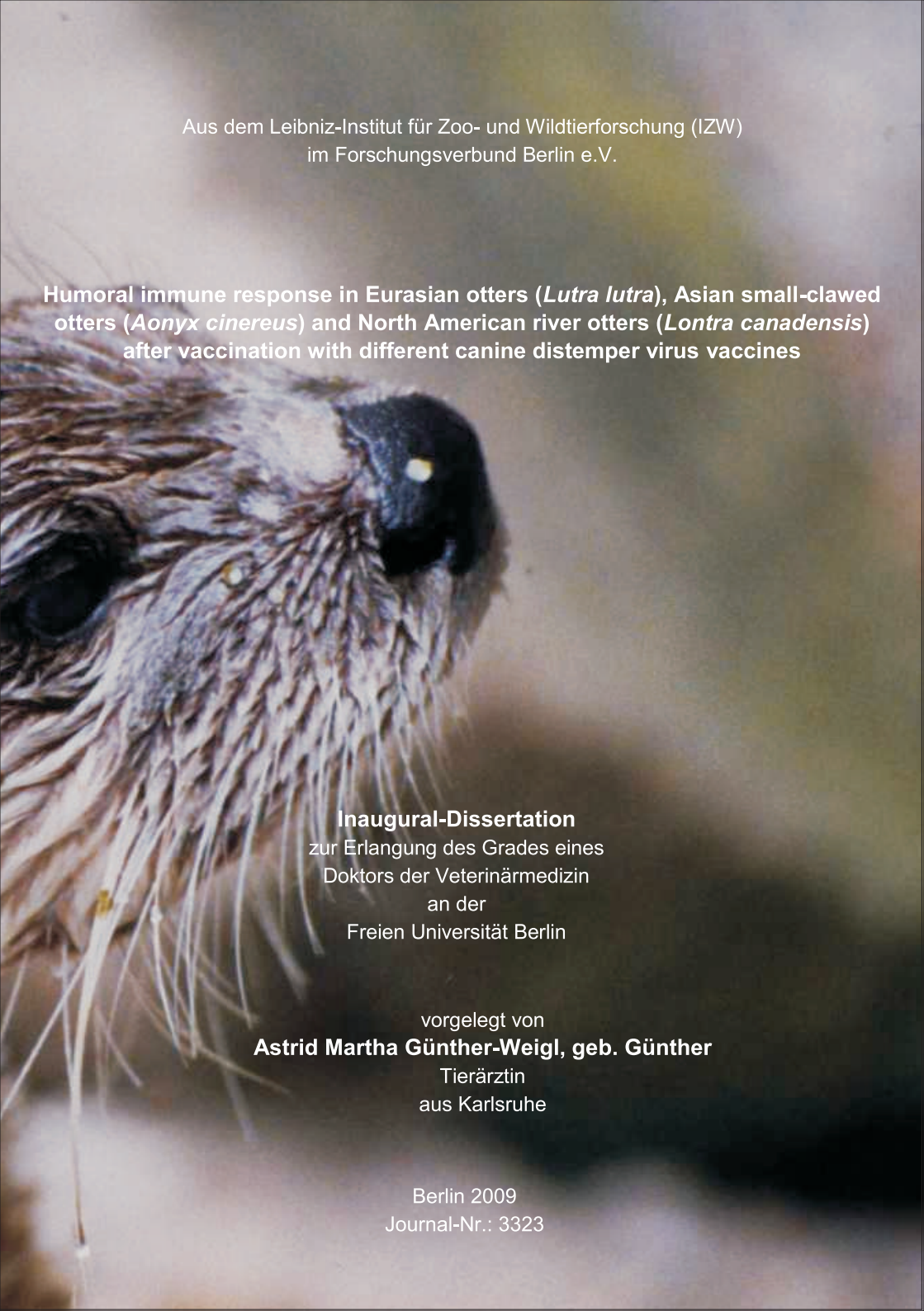
Aus dem Leibniz-Institut für Zoo- und Wildtierforschung (IZW)
im Forschungsverbund Berlin e.V.

Humoral immune response in Eurasian otters (*Lutra lutra*), Asian small-clawed otters (*Aonyx cinereus*) and North American river otters (*Lontra canadensis*) after vaccination with different canine distemper virus vaccines



Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Astrid Martha Günther-Weigl, geb. Günther
Tierärztin
aus Karlsruhe

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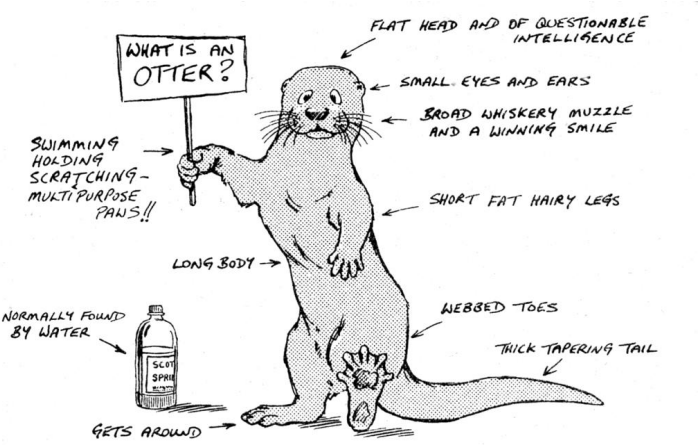
To Alicia, Ariella and Robert



An dem Fischotter ist alles merkwürdig, sein Leben, sein Treiben im Wasser, seine Bewegungen, sein Nahrungserwerb und seine geistigen Fähigkeiten. Er gehört unbedingt zu den anziehendsten Thieren unseres Erdtheiles.

(From Brehm`s Tierleben, Alfred Edward Brehm 1887 and Otters, Paul Chanin 1992. Illustration by Guy Troughton. Reprinted with permission of Whittet Books Ltd, Essex, England).

CONTENTS

ABBREVIATIONS

AAZV	American Association of Zoo Veterinarians
AK	Antikörper
$Al(OH)_3$	aluminium hydroxide
AMPV	avian metapneumovirus
APV	avian paramyxovirus
Aqua dest	aqua destillata
bp	nucleotide basepairs
BM	body mass
BU	Bussell
BPIV	bovine parainfluenzavirus
CAV	canine adenovirus
CD	canine distemper
CDV	canine distemper virus
cELISA	capture-sandwich enzyme-linked immunosorbent assay
CI	confidence interval
CNS	central nervous system
CPE	cytopathic effect
CPV	canine parvovirus
DMEM	Dulbecco´s modified eagle medium
DMV	dolphin morbillivirus
DNA	desoxy-ribonucleic acid
EDTA	ethylene-diamine-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMEA	European medicines agency
EU	European Union
F	fusion (-protein)
FCS	foetal calf serum
GMT	geometric mean titre
GMW	geometrischer Mittelwert
H	haemagglutinin (-protein)
HMPV	human metapneumovirus
H_2O_2	hydrogen peroxide
HPIV	human parainfluenzavirus
HRSV	human respiratory syncytialvirus
IF	immunofluorescent
IFT	immunofluorescence test
Ig	immunoglobulin
ISCOM	immuno-stimulating complex
IUCN	World Conservation Union
L	large (-protein)
LE	Lederle
M	matrix (-protein)
MLV	modified live virus

MV	measles virus
N	nucleocapsid (-protein
ND_{50}	neutralising dose 50% endpoint
NP	nucleoprotein
NPLAT	neutralising peroxidase-linked antibody test
NT	Neutralisationstest
OD	optical density
OP	Onderstepoort
P	phospho (-protein)
P	probability (statistical test)
PBS	phosphate buffered saline
PBST	phosphate buffered saline with Tween 20
PCR	polymerase chain reaction
p.i.	post infection
PDV	phocine distemper virus
PMV	porpoise morbillivirus
PPRV	pestes des petites ruminantes virus
RNA	ribonucleic acid
RO	Rockborn
RT-PCR	reverse transcriptase polymerase chain reaction
RV	rinderpest virus
SeV	Senai virus
Tc	cytotoxic T cell
$TCID_{50}$	tissue culture infectious dose 50% endpoint
Th	T helper cell
TMB	tetramethylbenzidine
UTR	untranslated region
VN	virus neutralisation
VNT	virus neutralisation test

1. INTRODUCTION

Canine distemper (CD) is one of the most important diseases of domestic dogs and other carnivores (Appel and Montali 1994). It is caused by the canine distemper virus (CDV), a morbillivirus closely related to measles virus of primates and pest des petites ruminants virus and rinderpest virus of ruminants. Recent emergence of CD in species not previously known to be naturally susceptible, including felids and marine mammals, and the significant impact of CD on some endangered species make CD an infectious disease of major concern for the management of free-ranging and captive carnivores (Montali et al. 1987; Williams and Thorne 1996).

All members of the order Carnivora are in principle susceptible to CDV (Philippa 2007) but morbidity and mortality greatly vary between different families and species. Highly susceptible are some members of the family Mustelidae (Deem 2000). Vaccination combined with preventive measures is the most effective way to prevent and control CD. Therefore, vaccination of mustelids held in captivity is highly recommended (Aiello 1998; Miller and Anderson 2000).

CDV vaccination in non-domestic carnivores has been problematic. Vaccine-induced CD has occurred in many carnivore species, including mustelids such as the domestic ferret (*Mustela putorius furo*), the black-footed ferret (*Mustela nigripes*) and the European mink (*Mustela lutreola*), when using modified live virus (MLV) vaccines. Furthermore, these vaccines have not always been effective in mustelids, including otters (Hoover et al. 1989; Goodrich 1994; Pavlacik 2007; Krüger, personal communication). Hence, safe and effective alternatives such as inactivated virus vaccines, subunit vaccines or recombinant vaccines are recommended (Montali et al. 1994). Currently, none of the safe alternatives are commercially available in Europe. The availible CDV vaccines are MLV vaccines registered for the application in domestic dogs, domestic ferrets or fur animals, and few CD vaccines have been tested for safety and efficacy in wildlife species.

CDV infections in captive breeding programs after vaccination have been devastating for endangered species such as the black-footed ferret (Carpenter et al. 1976; Pearson 1977) and the African wild dog (*Lycaon pictus*) (van de Bildt et al. 2002). The reasons for the observed incidents, vaccine-induced disease and vaccination failure, respectively, illustrates that the search for a safe and effective vaccine is of uttermost importance in CDV susceptible threatened or endangered wildlife species. Moreover, it suggests that ideally this search should include an extensive test phase with good experimental design, that extrapolating data from domestic animals to wildlife species is dangerous and that a safe and effective vaccine is needed for the protection of susceptible endangered species in captivity. The importance of effective vaccines against CD is further emphasized by the fact that the CDV has apparently enlarged its host species spectrum, leading to death in a variety of aquatic and terrestrial wild animals in the last 20 years (Appel et al. 1994; Harder et al. 1996; Osterhaus et al. 1988; Kock et al. 1998).

In zoos, many carnivore species susceptible to CDV live in close vicinity to each other. Few data are availible about the efficacy of CDV vaccines in otters, which are very common in

European zoos. The Eurasian otter (*Lutra lutra*) is listed as near threatened by the IUCN red list (October 2008), the Asian small-clawed otter (*Aonyx cinereus*) is listed as vulnerable, and in both species the free-ranging world population is decreasing. Also, the Eurasian otter and the North American river otter (*Lontra canadensis*) are part of re-introduction programs in many countries (Hoover 1985; Melissen 2000; Kimber 2000).

The objective of the present thesis was therefore to evaluate and compare the efficacy and safety of CDV vaccines available in Europe in different otter species to contribute to the conservation of these threatened wildlife species.

2. REVIEW OF LITERATURE

2.1 Canine distemper

Canine distemper (CD), a morbillivirus infection of dogs and other carnivores, has been recognised for at least 250 years. As reviewed by Blancou (2004), the first report of CD is from South America by Ulloa in 1746. Heusinger (1853) was convinced that CD was introduced from Peru to Spain in 1760, from where it spread to other parts of Europe and Russia within a few years. Although CD may have occurred in Europe earlier and was possibly confused with rabies, the epidemic spread of CD through Europe started around the 1760s. In 1815, Jenner described the contagiosity of the disease among dogs. But the etiology of CD remained controversial until 1905, when Henri Carré demonstrated that CD is caused by a filterable virus (Carré 1995a).

2.1.1 Taxonomy

Canine distemper is caused by the canine distemper virus (CDV), which belongs to the genus *Morbillivirus* in the family Paramyxoviridae. The family is subdivided into the subfamilies Paramyxovirinae and Pneumovirinae. Subdivision is based on differences in the second envelope glycoprotein and the number of encoded genes. CDV belongs to the subfamily Paramyxovirinae. Within this subfamily five genera, *Respirovirus*, *Rubulavirus*, *Morbillivirus* and the newly established genera *Avulavirus* and *Henipavirus* (Lwamba et al. 2005) have been described. Viral species of these genera are differentiated by the size and shape of their nucleocapsid, their antigenetic cross-reactivity, the presence or absence of neuraminidase activity and the coding potential of the phosphor (P) gene. The type virus of the genus *Morbillivirus* is measles virus. An overview of the family Paramyxoviridae is presented in Table 1.

2.1.2 Morphology

All morbilliviruses are closely related, which is reflected by the similarity of structure and genome. CDV virions are spherical in shape, 150-300 nm in diameter and consist of a non-segmented linear single-stranded RNA genome of negative polarity, 15 900 bp in size. The RNA is enclosed in a large (150-250 nm) helical nucleocapsid formed by the nucleocapsid protein (N). Mature ribonucleoprotein complexes (RNP) also contain copies of the phospho protein (P) and large protein (L). The host cell derived lipid envelope is spiked with the transmembrane hemagglutinin attachment protein (H) and the fusion protein (F). Both transmembrane proteins play a key role in the pathogenesis of all paramyxovirus infections. Internally, the envelope is stabilised by a layer of the matrix protein (M) (Figure 1). The gene order is generally conserved within the family: 3`-UTR (untranslated region) -N-P (C,V)-M-UTR-F-H-L-UTR-5`. The P gene encodes in addition to the P protein two non-structural proteins, the C protein and the V protein (Murphy et al. 1999; Griffin 2001).

Table 1 Paramyxoviridae – taxonomy, host spectrum, disease or clinical signs (adapted and modified from Osterhaus et al. 1995; Murphy et al. 1999; Lwamba 2005).

Subfamily/ Genus	Virus	Natural host	Disease or clinical signs
Paramyxovirinae			
Avulavirus	Avian paramyxovirus (APV-1)	Birds	Newcastle disease
	Avian paramyxovirus (APV-2…9)	Fowl	Respiratory disease
Rubulavirus	Mumps virus (MV)	Human	Mumps
	Human parainfluenza virus 2 (HPIV-2)	Human	Respiratory disease
Morbillivirus	Measles virus (MV)	Human	Measles
	Canine distemper virus (CDV)	**Carnivores**	**Canine distemper**
	Phocine distemper virus (PDV)	Seals	Phocine distemper
	Dolphin morbillivirus (DMV)*	Dolphins	Respiratory distress, abnormal behaviour
	Porpoise morbillivirus (PMV)*	Porpoise	
	Rinderpest virus (RV)	Cattle	Rinderpest
	Peste-des-petites-ruminantes virus (PPRV)	Goat, sheep	Pestes des petites ruminantes
Henipavirus	Hendravirus	Fruit bat, horse, human	Encephalitic syndrom
	Nipahvirus	Fruit bat, pig, human	Neurological and respiratory disease
Respirovirus	Sendaivirus (SeV)	Mouse, rat, guinea pig, hamster	Respiratory disease
	Human parainfluenza virus 1 and 3 (HPIV-1 and -3)	Human	Respiratory disease
	Bovine parainfluenza virus 3 (BPIV-3)	Cattle	Respiratory disease
Pneumovirinae			
Pneumovirus	Human respiratory syncytial virus (HRSV)	Human, primates	Respiratory disease
Meta-pneumo-virus	Human metapneumovirus (HMPV)	Human	Respiratory disease
	Avian metapneumovirus (AMPV)	Fowl	Respiratory disease

* DMV and PMV are currently gathered under the common denomination of “Cetacean morbilliviruses” (CMV)

2.1.3 Proteins

The **N protein** is the major component of the nucleocapsid and is produced at the highest concentration in infected cells. The N protein protects the RNA from degradation and is associated with the **P protein** which acts as cofactor for the **L protein**, the RNA-dependent RNA-polymerase. The RNP complex, made up of the RNA encapsulated by the N, P and L proteins, never disassembles during the infectious cycle (Lamb and Kolakofsky 2001).

The **H protein** is the most important protein for both CDV itself and its animal host, because it mediates the binding of the virus to the cell membrane of the host cell in the first step of infection (Appel 1987; Greene and Appel 2006; von Messling et al. 2001). Two clusters have been found which are responsible for receptor recognition (von Messling et al. 2005). Furthermore the H protein is the major determinant of CDV tropism, affects also fusion efficiency and thus contributes to cytopathogenicity (von Messling et al. 2001).

The **F protein** mediates membrane fusion which enables the entry of viral RNP into the cytoplasm (Lamb 1993). It is the major antigen against which neutralising antibodies are directed (Norrby et al. 1996). Variability among amino acid sequences of the mature F proteins from different CDV strains is about 4 %, which is in the range of variability of the other structural proteins whereas the CDV H proteins vary about 10 % (von Messling et al. 2001). The less pronounced variability among the amino acid sequences of the F protein from different CDV strains is probably due to their conserved function for protein folding, transport and fusion activity (von Messling and Cattaneo 2003).

The **M protein** is considered to be the central organizer of viral morphogenesis, being able to interact with the nucleocapsid core via the N protein as well as with the envelope glycoproteins via their cytoplasmatic tail (Takimoto and Porter 2004).

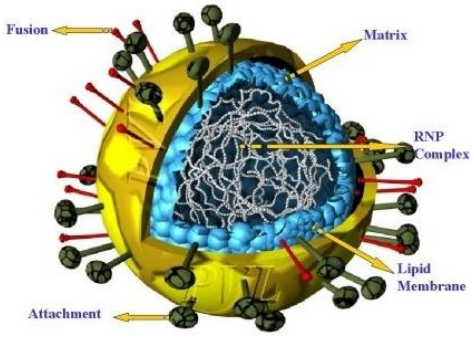


Figure 1 Structure of a morbillivirus: Fusion protein (F), attachment protein (H), matrix protein (M). From the universal virus database of the International Committee on Taxonomy of Viruses 2006 (www.ncbi.nlm.nih.gov./ICTVdb/index.htm).

2.1.4 Physical properties

CDV is relatively fragile and quickly inactivated in the environment by ultraviolet light as well as by heat and drying. It is destroyed by temperatures above 50°C in 30 minutes, but it can survive for 48 hours at +25°C. It may remain stable for weeks at 4°C and for years when frozen at -65°C (Appel 1987). Viral infectivity is lost above pH 9 or below pH 4. Common disinfectants readily inactivate CDV (Rolle and Mayr 2007).

2.1.5 Genotypes

Serologically, all morbillivirus species are considered monotypic. The classical methods, for example complement fixation test and immunofluorescence staining with polyclonal antibodies, do not distinguish serotypes among CDV strains. However, based on recent phylogenetic analysis of subgenomic F, P and complete H gene sequences, clustering of isolates became evident, which reflects geographic origin rather than host origin (Carpenter et al 1998; Lednicky et al. 2004; Lan et al. 2005; Martella et al. 2006). At least five to six separate clusters of wild-type CDV can be distinguished which differ by more than 0.5% at the nucleotide level (H gene). According to the definition used in the analysis of measles virus strain variation (Hsu et al. 1993) these clusters are referred to as genotypes (Figure 2). There is considerable biological variation among isolates of CDV. The genotypes differ in their pathogenicity which may affect the severity of clinical disease (Greene and Appel 2006). At the amino acid level, the greatest difference (10.2%) is observed between the H proteins of the Onderstepoort strain (vaccine strain) and a wild-type isolate from a Chinese leopard (Harder and Osterhaus 1997).

2.1.5.1 CDV vaccine strains

The CDV vaccine strains, which form a separate distinct lineage, originate from wild-type isolates made in the 1940s and 1950s (Greene and Appel 2006). The first CDV vaccine strains were „Onderstepoort" and „Rockborn". The Onderstepoort strain was developed in 1956 (Haig 1956). It was first grown in chicken embryos and later adapted to chicken-cell tissues. Because of its complete lack of virulence it is used as modified live virus (MLV) vaccine in domestic dogs (Haig 1948; Appel and Gillespie 1972) since the 1960s. The Onderstepoort strain is able to replicate in different cell lines, for example Vero and HeLa, and leads in most cases to cytolytic infection with pronounced formation of syncytia (Appel 1978). The „Bussell" strain (Bussell and Karzon 1965) is a Vero cell adapted clone of the Onderstepoort strain. The „Lederle" strain, which is used in several CDV vaccines for domestic dogs, is also chicken cell adapted.

The Rockborn strain, which was developed in 1958 (Rockborn 1959), is adapted to canine kidney cells and causes, in contrary to the Onderstepoort strain, no formation of syncytia. The Rockborn strain induces high titres of neutralising antibodies and long term protection. Unfortunately, this strain occasionally leads to vaccine-induced encephalitis in dogs and, more commonly, in exotic carnivores (Appel and Summers 1995; Greene and Appel 2006). The Onderstepoort strain may produce lower levels of immunity (Appel and Robson 1973) but will not induce disease in dogs, although chicken cell adapted vaccines have been also

fatal for different wildlife species (Greene and Appel 2006). A commonly used strain in vaccines is also the „Snyder Hill strain" which is indistinguishable from the Rockborn strain.

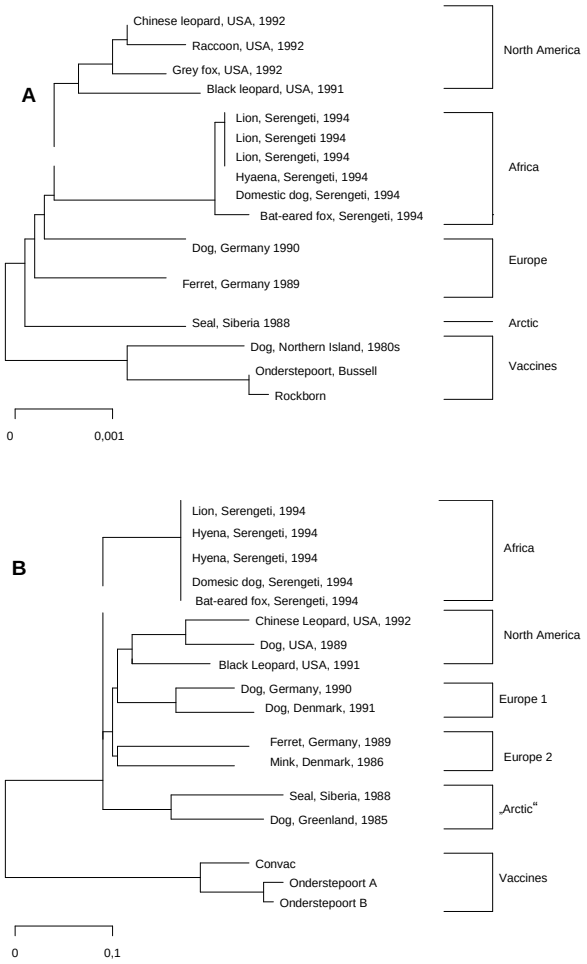


Figure 2 Phylogenetic relationship between canine distemper virus isolates based on (A) analysis of a 388bp fragment of the P gene and (B) on the entire coding region of the H gene and analysis of H gene fragments (Serengeti), respectively. Genetic distances (see bars for scale) were calculated by Kimura`s 2-parameter method. Host species, origin and year of isolation are indicated (modified from Harder and Osterhaus 1997 and Carpenter et al. 1998).

Virulent and attenuated CDV genotypes can be distinguished by their ability to replicate in epithelial cell cultures such as Vero cells. Vaccine strains easily replicate in these cells, whereas virulent wild-type strains require adapting (Evans et al. 1991; Appel et al. 1992). Another distinguishable trait is the neutralisation titre which is up to tenfold greater in sera raised against wild-type CDV isolates than against vaccine strains (Harder et al. 1996). Wild-type and vaccine strains can also be distinguished with monoclonal antibodies against their H proteins (Hamburger et al. 1991; Sheshberadaran et al. 1986).

2.1.6 Pathogenesis and clinical signs

Pathogenesis

Pathogenesis of CD is best studied in dogs and may be similar in non-domestic carnivore species (Appel 1969; Greene and Appel 2006; Deem 2000). Within 24 hours of entering the respiratory tract, viral spread occurs via macrophages to local lymphatics, tonsils and bronchial lymph nodes. Here the virus replicates 2-4 days post infection (p.i.), and proliferates widely into other lymphoid organs. Multiplication occurs from day 4-6 p.i. in the lymphoid follicles of the spleen, the lamina propria of the stomach and small intestine, and in the Kupffer cells in the liver, accompanied by an initial fever 3-6 days p.i.. Further spread to epithelial and central nervous system (CNS) tissues 8-9 at days p.i. depends on the immune status of the dog, and most likely takes place both as a cell associated and plasma phase-viremia (Appel 1987; Greene and Appel 2006).

Within the mustelid family the pathogenesis of CDV is well documented in ferrets. Ferrets are particularly sensitive to CDV and usually succumb to the infection without ever developing an effective immune response (von Messling et al. 2003). The initial phase is characterized by massive infection of the lymphatic organs, resulting in dramatic depletion of circulating lymphocytes. At day 7 p.i., most of the remaining circulating lymphocytes are infected, setting the stage for the invasion of epithelial tissues, including the upper and lower respiratory tract (von Messling et al. 2004). Subsequent disease phases include widespread epithelial infection followed by neuroinvasion. In ferrets, additional to the classical haematogenous CNS invasion pathway, anterograde invasion via the olfactory nerves was observed (Rudd et al. 2006).

In dogs, the course of CDV initially follows the same pattern, including the infection of cells in locations consistent with haematogenous invasion (Rima et al. 1991; Greene and Appel 2006). However, dogs with adequate antibody titres and cell mediated cytotoxicity (up to 90%) will clear the virus from most tissues without developing clinical signs (Rima 1991; Greene and Appel 2006; Tippold et al.1999). In ferrets and dogs that fail to mount an immune response the virus will spread to epithelial and central nervous tissues with concurrent development of typical clinical symptoms (Greene and Appel 2006; Rudd et al. 2006). Dogs with an intermediate cell-mediated immune response and delayed humoral response will have most viruses cleared as antibody titres rise. However, delayed CNS signs and hyperkeratosis of the foot pads (“hard pad disease”) may result when virus persists in uveal, neural and skin tissues (Greene and Appel 2006).

Clinical signs

Clinical signs of CD are influenced by virus strain virulence, environmental conditions, host age, immune status, and host species identity. In all susceptible species the respiratory, gastrointestinal, integumentary, and CNS system are most commonly affected. Biphasic fever and general malaise are often associated with viremia. Secondary infections are common and may often complicate the clinical course (Deem 2000).

Clinical signs in acute generalized CD are related to the respiratory and gastrointestinal system and include conjunctivitis, nasal discharge, dyspnoe, diarrhea (often haemorrhagic), anorexia, vomitus and severe dehydration. Neurological signs, which vary according the CNS areas involved, can coincide with the systemic signs, but usually begin one to three weeks after recovery from systemic illness, and are typically progressive. Neurological signs may emerge several months later without any preceding systemic signs (Greene and Appel 2006). Other signs of CD in domestic dogs include vesicular or pustular dermatitis in puppies, and nasal and digital hyperkeratosis ("hard pads"). CDV infection before the eruption of the permanent teeth may cause enamel hypoplasia characterized by irregularities in the dental surface (Greene and Appel 2006).

In mustelids, the first recognized clinical signs are serous oculonasal exudates, photophobia, hyperaemia and thickening of the eyelids, lips and anus (Budd 1981; Pearson and Gorham 1987). Secondary bacterial infection of the skin results in pruritus, especially of the face. Other clinical signs include fever, depression, respiratory signs, diarrhea, dehydration, anorexia, behavioural changes and convulsions (Williams and Thorne 1996). Black-footed ferrets often show severe hyperkeratosis of the foot pad, whole body erythema and chin and groin rash with associated pruritus (Williams et al. 1988; Carpenter et al. 1976). Ferrets experimentally infected with a virulent CDV strain developed full-body rash, starting at the mouth and skin region 6 to 8 days p.i., severe leukopenia and dehydration caused by diarrhoe, fever as well as respiratory signs such as pneumonia and purulent conjunctivitis. Between 12 and 16 days p.i. the animals were moribund and had to be euthanised (von Messling et al. 2003).

2.1.7 Epidemiology

Distribution, host range and susceptibility

CDV has a worldwide distribution. Its natural host range includes many species of carnivores (Figure 3), but morbidity and mortality greatly vary between families and species, ranging from sub-clinical infections in Hyaenidae and bears, up to 100% mortality in domestic ferrets and black-footed ferrets. In infected dogs 50-70% may remain asymptomatic carriers (Deem 2000; Appel et al. 2001).

In recent years, the host range of CDV appears to have widened due to interspecies transmission, leading to epizootics among pinnipeds and large cats with high mortality (Harder and Osterhaus 1997). Probably, the virus is able to infect all members of the order Carnivora (Phillipa 2007). Furthermore natural CDV infections have been observed in

Japanese macaques (*Macaca fuscata*) (Yoshikawa et al. 1989) and collared peccaries (*Tayassu tajacu*) (Appel et al. 1991). Other species can be infected experimentally with varying degrees of susceptibility (Appel and Summers 1995). Fatal experimental induced encephalitis has been documented in primates and pigs (Appel et al. 1974; Matsibara et al. 1985; Yamanouchi et al. 1977).

Transmission

CDV is most abundant in respiratory exudates and is commonly spread by aerosol or droplet exposure. However, it can also be isolated from most other body tissues and secretions. Transplacental transmission can occur from viremic dams. Virus shedding starts 7 days after infection and the virus might be excreted up to 60-90 days although shorter periods are more typical. Due to the relative fragility of CDV in the environment, close contact between recently infected (sub-clinical or clinical) and susceptible animals is necessary to maintain the virus in a population (Greene and Appel 2006). Fully recovered animals are not persistently infected, do not shed virus and are probably immune lifelong (Appel et al. 2001).

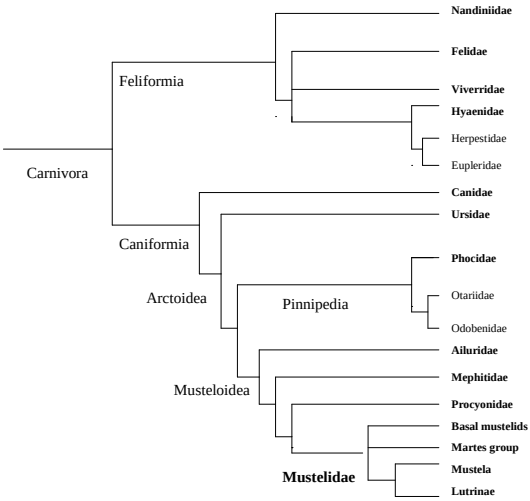


Figure 3 Phylogenetic tree of the families within the order Carnivora. The families with species reported to be susceptible to CDV are given in bold (adopted from Flynn et al. 2005, modified according to Appel and Summers 1995 and Deem et al. 2000).

Host-agent relationship

Epidemiology of CD depends on a variety of factors such as susceptibility of species, population density and intraspecific as well as interspecific behaviour that influences transmission. In areas with endemic CD in domestic dogs and a high domestic dog

population, clinical disease is mostly seen in pups after maternal antibodies drop at 3-6 months of age. In isolated dog populations, CD occurs in epidemics, and outbreaks may be severe and widespread with domestic dogs at all ages affected (Leighton et al. 1988; Bohm et al. 1989).

Despite the wide host range, domestic dogs are regarded as the principal reservoir host for CDV and they likely act as reservoir for wildlife infection (Harder and Osterhaus 1997). Certain wildlife species such as raccoons (*Procyon lotor*) in North America (Hoff und Bigler 1974; Mitchel et al. 1999; Roscoe 1993) or masked palm civets (*Paguma larvata*) in Japan (Machida et al. 1993) might serve as a reservoir of infection for susceptible domestic dog populations. Since the introduction of MLV vaccines, the disease has been under control in domestic dogs. However, because of widespread CD outbreaks in free-ranging carnivores, eradication does not seem possible (Appel et al. 2001). In addition, insufficient vaccination and/or vaccines with low efficacy in certain areas can cause severe disease outbreaks in domestic dog populations (Ek-Kommonen et al. 1997; Blixenkrone-Møller et al. 1993; Bohm et al. 1989).

2.1.8 Canine distemper in non-domestic species

CD was recognized in captive non-domestic species for the first time around 1905. About 50 years later it was reported in free-ranging wildlife (Helmboldt and Jungherr 1955). The already wide host range of CDV (Canidae, Mustelidae, Procyonidae, Hyaenidae, Ursidae, Viverridae) recently expanded with occurrence of epidemics in free-ranging felids (Feldidae) and marine mammals (Phocidae) which had not been previously known to be susceptible to natural CDV infection (Williams 2001). Today, natural infection of CDV has been documented in nearly all families of terrestrial and aquatic carnivores (Table 2 and 3, Figure 3), as well as in primates and peccaries (reviewed in Appel et al. 2001; Williams 2001; Philippa 2007).

In North America, coyotes (*Canis latrans*), wolves (*Canis lupus*) and raccoons are common canid hosts of CDV. Surveys and diagnostic reports demonstrated that red foxes (*Vulpes vulpes*) are susceptible to CD but appear to be more resistant than the highly susceptible grey fox (*Urocyon cinereoargenteus*). Natural CD in red foxes was shown by a two year study on 236 carnivores (146 mustelids, 90 red foxes) in Germany by van Moll et al. (1995). Among African canids CD was reported from African wild dogs and two jackal species (*Canis mesomelas*, *Canis adustus*) (Alexander et al. 1995). In Asia, CD was observed in free-ranging raccoon dogs in Japan (Machida et al. 1993). Lesser pandas (*Ailurus fulgens*) are highly susceptible to CDV (Kotani et al. 1989) and there are numerous reports of vaccine-induced disease in this species (Bush et al. 1976; Montali et al. 1987).

According to serological surveys, many ursids are susceptible to CDV infection but clinical disease appears to be rare (Williams et al. 2001; Deem 2000). There is one report of CDV in captive polar bears (*Ursus maritimus*) and a spectacled bear (*Tremarctos ornatus*) from Europe (von Schönbauer et al. 1984). Clinical CD has most commonly been documented in free-ranging and captive giant pandas (*Ailuropoda melanoleuca*) in China (Qui and Mainka 1993; Mainka et al. 1994).

The first CD epizootic outbreak among large cats occurred in Wildlife Waystation in San Fernando, California, in 1992, where 17 lions (*Panthera leo*), tigers (*Panthera tigris*) and leopards (*Panthera pardus*) died from CD (Appel et al. 1994). The second outbreak was reported from Serengeti National Park, Tanzania in 1994 where an estimated 30% of the free-ranging lion population died. Hyenas, jackals, bat-eared foxes (*Otocyon megalotis*) and leopards were also affected (Roelke-Parker et al.1996). However, CD seems to be not a new disease in lions and tigers. A retrospective study conducted in Switzerland using necropsy cases from 42 captive lions and tigers, which died between 1972 and 1992, yielded 19 positive and 23 negative or questionable findings by immunohistochemistry, suggesting that CDV infection of large cats is older and more widespread than previously thought (Myers et al. 1997).

CDV has become established as a disease in aquatic environments, in both marine and freshwater habitats (Barrett 1999). It has caused fatalities in thousands of Baikal seals (*Phoca sibirica*) in Lake Baikal in Russia from 1987-1988 (Osterhaus et al. 1989a; Grachev et al. 1989) as well as in Caspian seals (*Phoca caspica*) in the Caspian Sea from April to August 2000 (Kennedy et al. 2000). CDV antibodies have been found in different marine mammals including harp seals (*Phoca groenlandica*), hooded seals (*Cystophora cristata*) and leopard seals (*Hydrurga leptonyx*) (Stuen et al. 1994; Bengtson et al. 1991). Furthermore CDV might have been involved in mass mortality among crab-eating seals (*Lobodon carcinophagus*) in Antarctica in 1955 (Bengston et al. 1991). Species (excluding mustelids) reported to be susceptible to canine distemper virus are shown in Table 2.

Table 2 Species reported to be susceptible to canine distemper in the order Carnivora excluding mustelids. Evidence of susceptibility to CDV in free-ranging (f) or captive (cp) wildlife species was provided by clinical (c) or pathological (p) findings, by virus isolation (v), RT-PCR (pc) with consecutive nucleotide sequence analysis (ns) or serological methods (s). n.r. = not reported.

Family	Species	Scientific name	Evidence	Reference
Ailuridae	Red panda (cp)	*Ailurus fulgens*	c, p, v	Bush et al. 1976
Canidae	Red fox (f)	*Vulpes vulpes*	s	Amundson and Yuill 1983
			s, ns	Frölich et al. 2000
	Kit fox (f)	*Vulpes macrotis mutica*	s	McCue and O´Farell 1988
	Fennec fox (cp)	*Vulpes zerda*	n.r.	Coke et al. 2005
	Artic/blue fox (f)	*Alopex lagopus*	n.r.	Rausch 1953
	Grey fox (f)	*Urocyon cinereoargentus*	c, p	Davidson et al. 1992
	Santa Catalina island fox (f)	*Urocyon littoralis catalinae*	c, p, pc, ns, s	Timm et al. 2009

Family	Species	*Scientific name*	Evidence	Reference
Canidae	Santa Catalina island fox (f)	*Urocyon littoralis catalinae*	s	Clifford et al. 2006
	Raccoon dog (f)	*Nyctereutes procyonoides*	c, p	Machida et al. 1993
	Bat-eared fox (f)	*Otocyon megalotis*	c, v	Roelke-Parker et al. 1996
			pc, ns	Carpenter et al. 1998
	Pampas fox (f)	*Pseudalopex gymnocercus*	s	Fiorello et al. 2007
	Crabeating fox (f; cp)	*Cerdocyon thous*	c, p,	Cubas 1996
			s	Fiorello et al. 2007
			c, p, pc, ns	Megid et al. 2009
	Maned wolf (cp)	*Chrysocyon brachyurus*	c, p	Cubas 1996
	Wolf (f)	*Canis lupus*	s	Choquette and Kuyt 1974
			s	Santos et al. 2009
	Australian dingo (cp)	*Canis lupus dingo*	c, p	Armstrong and Anthony 1942
	Coyote (f)	*Canis latrans*	s	Gese et al. 2004
			s	Bischof and Rogers 2005
	African wild dog (cp)	*Lycaon pictus*	c, p, v, pc, ns, s	van de Bildt et al. 2002
	Silver-backed jackal (f)	*Canis mesomelas*	s	Alexander et al. 1994
			s	Spencer et al.1999
	Side-striped jackal (f)	*Canis adustus*	s	Alexander et al. 1994
			s	Spencer et al. 1999
Felidae	African lion (f))	*Panthera leo*	c, p, s	Roelke-Parker et al. 1996
	Tiger (cp)	*Panthera tigris*	c, p, v, s	Appel et al. 1994
	Leopard (cp)	*Panthera pardus*	c, p, v, s	Appel et al. 1994
	Jaguar (cp)	*Panthera onca*	c, p, v, s	Appel et al. 1994
	Ocelot (f)	*Leopardus pardalis*	s	Fiorello et al. 2007
	Canadian lynx (f)	*Lynx canadensis*	c, p, pc, ns, s	Daoust et al. 2009
	Bobcat (f)	*Lynx rufus*	c , p, pc, ns, s	Daoust et al. 2009
Hyaenidae	Spotted hyena (f)	*Crocuta crocuta*	s	Alexander et al 1995
			c, p, v, pc, ns	Haas et al. 1996

Family	Species	Scientific name	Evidence	Reference
Mephitidae	Striped skunk (f)	*Mephitis mephitis*	c, p	Diters and Nielsen 1978
Phocidae	Baikal seal (f)	*Phoca sibirica*	c, p, s	Grachev et al. 1989
			c, p, s	Osterhaus 1989a
	Caspian seal (f)	*Phoca caspica*	s, pc, ns	Kennedy et al. 2000
	Saima seal (n.r.)	*Phoca hispida saimensis*	s	Rikula 2008
	Harp seal (f)	*Phoca groenlandica*	s	Stuen et al. 1994
	Hooded seal (f)	*Cystophora cristata*	s	Stuen et al. 1994
	Crabeater seal (f)	*Lobodon carcinophagus*	s	Bengtson and Boveng 1990
Procyonidae	Raccoon (f)	*Procyon lotor*	c, p, v	Roscoe 1993
			s	Raizmann et al. 2009
	Kinkajou (cp)	*Potos flavus*	c, p, s	Kazakos 1981
	South American coati (cp)	*Nasua nasua*	c, p, s	Cubas 1996
Ursidae	Black bear (f)	*Ursus americanus*	s	Dunbar et al. 1998
			s	Chomel et al. 2001
	Grizzly bear (f)	*Ursus arctos horribilis*	s	Chomel et al. 2001
	Marsican brown bear (f; cp)	*Ursus arctos marsicanus*	s	Marsilio et al. 1997
	Polar bear (cp; f)	*Ursus maritimus*	v	von Schönbauer et al. 1984
			s	Follmann et al. 1996
			s	Cattet et al. 2004
	Spectacled bear (cp)	*Tremarctos ornatus*	v	von Schönbauer et al. 1984
	Giant panda (f; cp)	*Ailuropoda melanoleuca*	c, p	Qui and Mainka 1993
			s	Mainka et al. 1994
Viverridae	Binturong (cp)	*Arctictis binturong*	c, p	Goss 1948
			c, p	Hur et al. 1999
	Masked palm civet (f)	*Paguma larvata*	c, p	Machida et al. 1992
	Common genet (f)	*Genetta genetta*	c, p	Lopez-Peña et al. 2001

In **mustelids,** CD has been reported in many parts of the world. In North America black-footed ferrets are highly susceptible (Williams et al. 1988), whereas striped skunks (*Mephistis mephistis*) appear to be more resistant (Dieters and Nielsen 1978). Reports of CD in European mustelids, includes epidemics in stone marten (*Martes foina*), polecat (*Mustela putorius*), European badger (*Meles meles*) and weasel (*Mustela sp.*) (Kölbl et al. 1990; Alldinger et al. 1993; van Moll et al. 1995).

Not many published data exist about CDV in otters. Wide scale epidemics in otters are not known, which is possibly due to the solitary lifestyle of most of the 13 known otter species. Otters held in captivity seem to be at more risk. Many zoos report about serologically confirmed CD in otters when asked. Clinically CD has been reported in North American river otters (Kimber et al. 2000; Mos et al. 2003), Eurasian otters (Geisel 1979; Loupal et al.; Madsen et al. 1998,1999) and Asian small-clawed otters (Bosschere et al. 2005) (Table 3).

Table 3 Mustelids reported to be susceptible to CDV. Evidence of susceptibility to CDV in free-ranging (f) or captive (cp) mustelids was provided by clinical (c) or pathological (p) findings, by virus isolation (v), RT-PCR (pc) with consecutive nucleotide sequence analysis (ns) or serological methods (s). n.r. = not reported.

Species	Scientific name	Evidence	Reference
American badger (f)	*Taxidea taxus*	s	Goodrich et al. 1994
Eurasien badger (f)	*Meles meles*	c, p,	Kölbl et al. 1990
		c, p,	Hammer et al. 2004
Ferret badger (f)	*Melogale moschata*	c, p, pc	Chen et al. 2008
European mink (cp; f)	*Mustela lutreola*	c, p, pc, ns	Ek-Kommonen et al. 2003
		s	Philippa et al. 2008
American mink (f)	*Mustela vison*	s	Pearson and Gorham 1997
		s	Philippa et al. 2008
Eurasian otter (cp; f)	*Lutra lutra*	c, p,	Geisel 1979
		p	Madsen et al. 1999
		s	Müller and Tschirsch 2001
		c, p,	Loupal et al. 2002
North American river otter (f; cp)	*Lontra candensis*	s	Kimber et al. 2000
		c, p, pc	Mos et al. 2003
Asian small-clawed otter (cp)	*Aonyx cinereus*	c, p,	de Bosschere et al. 2005
Black-footed ferret (f)	*Mustela nigripes*	c, p, v	Williams et al. 1988
Domestic ferret (cp)	*Mustela putorius furo*	s	Stephensen et al. 1997
		s	Welter et al. 2000
Stone marten (f)	*Martes foina*	c, p	Steinhagen and Nebel 1985
		p	van Moll 1995
		s, ns	Frölich et al. 2000
Sable (n.r.)	*Martes zibellina*	s	Rikula 2008

Species	Scientific name	Evidence	Reference
Polecat (f)	*Mustela putorius*	p	van Moll 1995
		s	Philippa et al. 2007
Pine marten (cp; f)	*Martes martes*	s	Rikula 2008
		s	Philippa et al. 2008
Wolverine (f)	*Gulo gulo*	s	Dalerum et al. 2005

2.1.9 Immune response

Immune response to vaccination depends on the same mechanisms as other immune responses (Selbitz and Moos 2006). In case of CDV vaccination, immune reaction is based on antibody production (IgM, IgG) directed against viral proteins (humoral response), on cytotoxic T cells (cell-mediated immune response) specific for various viral protein targets (Appel 1987; Greene and Appel 2006) and on production of memory cells (B and T memory cells) which form a reserve of long-lived antigen-sensitive cells (Tizard 2004) to be called on during subsequent exposure to antigens (Figure 4).
Humoral immune response is usually used as an indicator to assess vaccine efficacy and immune status of animals (Coyne et al. 2001; Twark and Dodds 2000). However, humoral immunity is not sufficient to explain resistance to canine distemper and many other virus infections (Appel and Summers 1995; Carmichael 1997). Cell-mediated immunity has a paramount importance not only in CDV infection but in all virus and morbillivirus infections, respectively. In human measles, individuals with agammaglobulinemia can overcome the infection but those with inherited or acquired deficiencies in their cell-mediated immune system develop a complicated and often fatal course of the disease (Murphy et al. 1999; Griffin and Bellini 1996). Especially cytotoxic T cells (Tc) against the N protein of CDV are considered to be crucial for protection against CDV infection (Cherpillod et al. 2000).

Humoral immunity can be demonstrated by measuring the titre of virus neutralising (VN) antibodies against CDV in serum. These antibodies are directed against the viral glycoproteins H and F (Appel 1987). The presence and titre of VN antibodies correlate well with the level of protection against CD in dogs (Norrby et al. 1996). The antibodies can be detected 6-10 days post vaccination in the blood serum. Maximum levels are reached after 14-21 days (Appel 1969 and 1987). Vaccination also elicits antibodies against other viral proteins, but the role of these in protective immunity is inconclusive (Rikula 2007).The N protein is the immuno-dominant morbillivirus protein and induces the most vigorous antibody response (von Messling et al. 1999). Antibodies to N and P proteins may appear after 6-8 days (Greene and Appel 2006).

As challenge infections are inappropriate in endangered species, protective titres in otters are not known (Ludwig Haas, personal communication). Puppies of domestic dogs with maternal VN antibody titres higher than 1:100 were protected against CDV infection (Gillespie 1996). Susceptible domestic dogs that developed titres of at least 1:100 by day 14 after challenge with virulent CDV survived (Appel 1969). According to Greene and Appel (2006), a VN antibody titre of 1:20 is considered protective in domestic dogs after

vaccination. Domestic ferrets with a VN titre between 1:172 and 1:512 after vaccination with a chicken-cell adapted MLV vaccine survived challenge infections but were not protected against clinical and laboratory signs of CD (Stephensen et al. 1997)).

Initially, mainly IgM antibodies are produced (Figure 4) which are then replaced by IgG antibodies after class switch (Noon et al. 1980; Winters et al. 1983; Appel 1987). A primary IgM response was detected by ELISA in minks 4 days after vaccination with an attenuated modified live virus CDV vaccine and 6 days after inoculation with a virulent strain of CDV (Blixenkrone-Møller et al. 1991). In contrast to immune response after natural infection, revaccination of CDV in dogs will not be followed by new IgM antibody response. Only IgG antibodies will be produced after second immunisation (Appel et al. 1987; Blixenkrone-Møller et al. 1991). In dogs, virus specific IgM is measurable for up to three weeks after first immunisation with CDV vaccine and up to three month p.i. (Greene and Appel 2006).

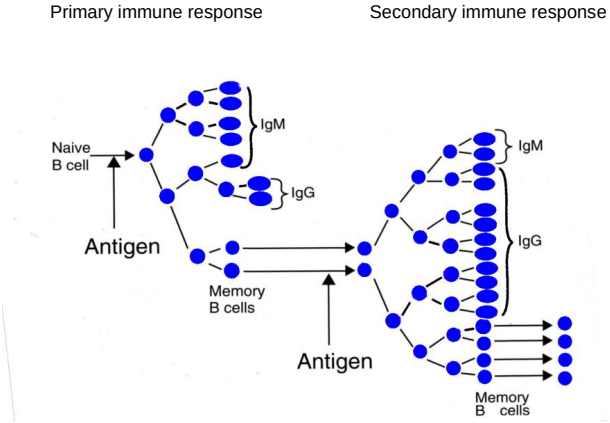


Figure 4 Humoral immune response and production of memory cells as the basis of vaccination success. Note how primary immune response stimulates mainly the production of IgM antibodies, whereas in a secondary immune response mainly IgG antibodies are induced. In contrast to IgM and IgG antibodies with half lives of 5 days and 20 days, respectively, memory cells are extremely long-lived (up to decades in humans), probably due to their resistance to apoptosis. For vaccination success, the production of long-lived T memory cells (additional to B memory cells) is of crucial importance (adapted and modified from Tizard 2004 and Selbitz and Moos 2006).

After recovery from natural CDV infection or repeated vaccination with MLV vaccine, virus specific IgG will last in dogs as well as in wild animals for perhaps their entire life or may at least persist for more than 6 years. This protection may be adequate unless the animal is exposed to a highly virulent virus strain or large quantities of virus or becomes stressed or immuno-compromised (Williams 2001; Greene and Appel 2006; Appel 1987). Infection with

measles virus, a closely related paramyxovirus, confers life-long immunity (Brookes et al. 2007).

Modified live virus vaccines induce immune responses which are in principle very similar to those occurring after natural infection. However, vaccine-induced immunity will never be as long lasting as the immune response that occurs after natural or experimental infection with virulent virus. Despite changes in the H protein of wild-type CDV strains, it is however, unlikely that virulent CDV strains can break through solid MLV vaccine induced immunity (Greene and Appel 2006). As the different vaccine strains and vaccines produce different levels of protection, the outcome of vaccination depends on the properties of the vaccine strain, on the formulation of the vaccine, and on several other factors such as individual variations, immune status, health status, epidemic situation, season and climate (Rikula 2001; Selbitz and Mos 2006).

Several methods have been used to measure **cellular immunity** against CDV (Appel et al. 1994b; Krakowka and Wallece 1979; Clough and Roth 1995). Widely and successfully used methods to measure antigen-specific cell-mediated immune responses against numerous infectious agents in animals, are lymphocyte proliferation assays (Clough and Roth 1995). Animals that have developed cell-mediate immunity against a specific antigen have an increase in the number of circulating lymphocytes that recognize the antigen. In proliferation assays, radioactive (tritiated) thymidine is added to lymphocytes being cultured. Actively dividing lymphocytes will incorporate the radioactive thymidine into new DNA molecules. The ratio of radioactivity in antigen-exposed lymphocytes to that in non-antigen exposed lymphocytes from the same animal provides an assessment of the developed cellular immunity (Clough and Roth 1995). Virus-specific cell-mediated immunity can be demonstrated from day 10-14 p.i. in circulating virus-specific Tc cells with a maximum at day 14-21 p.i. (Appel et al. 1982). However, the use of this method is hampered by the considerable expertise and equipment required, and is therefore not recommended to be used routinely for measuring immunity after vaccination (Clough and Roth 1995).

2.1.10 CDV diagnosis

Exposure to canine distemper virus can be confirmed by detecting either CDV itself or specific antibodies against the virus (Greene and Appel 2006).

2.1.10.1 Virus detection

Direct detection of CDV from smears of the conjunctival, tonsillar, genital or respiratory epithelia using immunofluorescent (IF) techniques is possible only within the first three weeks p. i., when systemic illness is apparent. As antibody titres rise in association with clinical recovery, the virus will either be masked by antibodies or will disappear from the epithelia. The sensitivity of the IF technique is ≤ 40 % (Blixenkrone-Møller et al. 1993; Leisewitz et al. 2001). Immunohistochemistry can be used to demonstrate CDV antigens in foot pad and skin biopsies or in samples taken post mortem from many tissues, among others from the spleen, tonsils, lymph nodes, bladder, brain, stomach, duodenum and other sections of the intestinum (Thijs Kuiken, personal communication; von Messling et al. 2001; Greene and

Appel 2006). The reverse-transcriptase-polymerase chain reaction (RT-PCR) has been used to detect CDV RNA in buffy coat cells from domestic dogs with acute CDV infection (von Messling et al. 1999) and from blood-serum, whole blood and cerebrospinal fluid of domestic dogs with systemic or neurological distemper (Shin et al. 1995; Frisk et al. 1999; Saito et al. 2006). In general, a positive PCR result is indicative of infection, whereas a negative PCR reaction may result from many factors, including improper sample handling (Greene and Appel 2006). In other words: PCR does not produce false positives but may produce false negatives.

Virus isolation can be difficult, as virulent CDV requires adaptation before it grows in routinely used epithelial or fibroblast cell lines. CDV Isolation was usually done by direct cultivation of buffy coat cells or other target tissues from the infected host together with mitogen-stimulated domestic dog lymphocytes (Greene and Appel 2006), although cytopathic effect (CPE) is difficult to detect in these cells (Seki et al. 2003). The best results are nowadays achieved by using Vero cells expressing canine signalling lymphocyte activation molecules (Vero.DogSLAM) (Thijs Kuiken, personal communication; Seki et al. 2003; Woma and van Vuuren 2009).

2.1.10.2 Antibody detection

CDV infection or CDV vaccination success can be confirmed by demonstrating specific antibodies to the agent (Greene and Appel 2006).

A four-fold rise in the antibody level of paired sera taken 10-21 days apart is indicative of infection. However, as antibodies are often high at the first sampling (infection already in advanced stage), a four-fold rise cannot always be demonstrated. Detection of CDV-specific IgM is indicative of a recent infection or vaccination high IgG titers indicate past or present infection or vaccination against CD. Increased CDV antibodies in cerebrospinal fluid offer definitive evidence of distemper encephalitis because antibodies are locally produced, provided the blood-brain barrier is intact (Greene and Appel 2006).

Virus Neutralisation test

The virus neutralisation test (VNT) is considered to be the gold standard for the detection and quantification of antiviral antibodies (Murphy et al. 1999). Neutralising serum titres correlate well with the level of protection (Appel and Robson 1973; Carmichael 1997; Coyne et al. 2001). Neutralisation tests estimate the ability of antibodies to neutralise the biological activity of antigen when mixed with it in vitro. Virus may be prevented from infecting cells after the specific antibody has bound and blocked their critical attachment sites. This reaction is the basis of the neutralisation test which is therefore highly specific and extremely sensitive (Tizard 2004). Analysis of the test is made by microscope, using the effect of giant cell formation (syncytia), a characteristic CPE of CDV in many tissues which is detected 2-5 days after tissue infection (Greene and Appel 2006).

Neutralising peroxydase-linked antibody test

To simplify analysis of the neutralisation test, virus-infected cells can be made visible with fluorescence-labelled antibodies or with peroxidase-linked antibodies. Zaghawa (1990) used a goat-anti-mouse-IgG-peroxydase conjugate to mark virusinfected cells. This test, referred to as “neutralising peroxydase-linked antibody test” (NPLAT), reduces the time to analysis from 5-7 days (without dying) to 3 days.

Enzyme-linked immunosorbent assays

In contrast to the VNT, enzyme-linked immunosorbent assays (ELISA) consider not only virus neutralising antibodies but all virus specific antibodies. They are performed by allowing antigen and antibody to bind, followed by measuring the amount of immune complexes formed. Radioisotopes, fluorescent dyes, colloidal metals and enzymes are used as labels to identify one of the reactants. ELISAs may be used to measure either antibody or antigen.

There are different types of ELISAs. The most common form, an indirect ELISA for antibody detection, uses micro-wells on polysterene plates coated with antigen. Presence of bound antibody in added serum is detected by means of enzyme-labelled antiglobulin (Tizard 2004; von Messling 1999; Örvell 1985) or enzyme-labelled protein A (Philippa 2007). Protein A is a surface protein originally found in the cell wall of *Staphylococcus aureus*. It binds with high affinity to the base region of IgG antibodies of most mammalian species (Fournier and Klier 2004) in a non-immunological reaction without interfering with the antigen binding sites (Stöbel et al. 2002). This property permits the formation of complexes consisting of protein A, antibody and antigen and has long been used for many preparative and analytical purposes in immunology (Akerström et al. 1985). Commercially available protein A is suitable as a second antibody for a great range of wildlife species, including mustelids, and is a useful alternative to species-specific secondary antibodies in various diagnostic assays (Stöbel et al. 2002).

Whole-virus indirect ELISA has proved to be a rapid and reliable method for serological survey of CDV infection in domestic dogs (Gemma 1995). A modification of this technique is the so-called sandwich ELISA. This test involves the formation of antibody-antigen-antibody layers or antigen-antibody-antigen layers. Different sandwich ELISAs have been developed for the detection of CDV antigen or antibodies in domestic dog sera by Potgieter and Ajidagba (1989) and all ELISA systems showed excellent sensitivity and specificity. A capture-sandwich ELISA (cELISA) that uses recombinant baculovirus–expressed CDV N protein was developed by von Messling et al. (1999). The cELISA showed rapid and sensitive detection of IgM and IgG antibodies against CDV and proved to be superior to VNTs with respect to sensitivity and specifity. A competitive ELISA developed for the differentiation between antibodies of CDV and PDV (Saliki and Lehenbauer 2001) also showed high sensitivity and specificity. By the use of an IgM or IgG specific conjugate, differentiation of different antibodies (IgM, IgG) is possible (King et al. 1993).

Immunofluorescence assays

The indirect fluorescence antibody test may be used to detect either antibodies or antigen. The antigen, in a tissue section, smear or culture will bind antibody from serum. After washing this antibody may be detected by binding to fluorescein isothiocyanate (FITC)-labelled antiglobulin (Tizard 2004). With an immunofluorescence assay, all virus specific antibodies are determined. This test has been used to measure post vaccination titres and gives results comparable to those of neutralisation (Twark and Dodds 2000).

Western Blotting

Western blot analysis may also be used to detect CDV specific antibodies. It is a three-stage primary binding test, involving serum separation by electrophoresis, transfer of the protein to nitrocellulose paper and subsequent visualisation by means of enzyme-immunoassay or radio-immunoassay (Tizard 2004). A Western blot analysis for the detection of CDV-specific dog IgM is described by Barben et al. (1999). The study showed that the IgM detection test is a useful method for diagnosing current or recent CDV infection in CDV-infected or CDV-immunised domestic dogs under experimental conditions.

2.1.11 Prevention and Control

Vaccination remains the most important tool to prevent and control CDV infections in domestic dogs, farmed fur animals, domestic ferrets kept as pets and other susceptible wildlife species kept in zoos (Appel and Summers 1995). Maintenance of high vaccination rates using efficacious vaccines that induce a solid resilient immunity must still be given the highest priority for the control of distemper, particularly in areas with high densities of domestic dogs, and their possible exposure to wild carnivores (Harder and Osterhaus 1997).

Domestic dogs infected with CDV should be isolated from healthy ones (Greene and Appel 2006). Furthermore, as no vaccination strategy can eliminate the gap in protection between passive maternal immunity and active immunity, prophylactic measures should include the isolation of young domestic dogs until vaccine-induced protection has been achieved (Blixenkrone-Møller et al. 1993).

As recent CDV epizootics have demonstrated that small populations of highly endangered species may be seriously affected by CDV epidemics, vaccination of free-ranging wild carnivores can be considered as an option (Harder and Osterhaus 1997; Greene and Appel 2006), provided there is good evidence that vaccination is safe and efficacious in the affected wildlife species and that the necessary vaccination cover can be realistically achieved. In addition to vaccination, strict biosecurity measures such as isolation and quarantine procedures for animals in fur farms and zoos (Pearson and Gorham 1987) are necessary as well as proper hygiene management, parasite control and adequate nutrition to maintain the immune system viable. Besides that, early detection of outbreaks by improved surveillance in susceptible species could make an important contribution to the detection and control of emerging infections (Kuiken et al. 2003, 2005).

2.2 Canine distemper vaccination

Vaccination has proved to be by far the most efficient and cost-effective method for the controlling of infectious diseases in humans and animals. Control of canine distemper in domestic dogs would not have been possible without the use of effective vaccines (Tizard 2004). Active immunisation against CD has been practised since Putoni (1923) described the use of formalin-inactivated CDV-infected dog brain tissue (reviewed by Appel 1999). However, active immunization was not successful before MLV vaccines became available in the 1950s.

Vaccination of CDV-susceptible wild carnivores in zoos has been recommended since 1963 (Christensen 1963). CDV vaccination is recommended in all members of the families Canidae, Procyonidae and Mustelidae (Aiello 1998; Miller and Anderson 2000). After several outbreaks of CD among captive and free-ranging large felids (Appel et al. 1994; Harder et al. 1995, 1996; Roelke-Parker et al. 1996) vaccination of large cats is suggested in high risk situations (Aiello 1998, Kennedy-Stoskopf 1996; Miller and Anderson 2000). The need for vaccination of the species of the Ursidae, Hyaenidae and Viveriidae is discussed (Aiello 1988; Miller and Anderson 2000), as clinical disease and presence of CDV-specific antibodies have been shown in different species of these families (Machida 1992; Alexander et al. 1995; Haas et al. 1996; Cattet et al. 2004).

2.2.1 Principles of vaccination

The principle objective of vaccination is to induce an immune response that mimics protection acquired after natural infection. An ideal vaccine would therefore induce a strong virus neutralising serum antibody response with high titres of long duration, would induce T-cell mediated immunity as well as mucosal immunity, and would be free of side effects. Moreover, this vaccine would be cheap, stable, suited for mass vaccination and would stimulate an immune response distinguishable from that of natural infection (Tizard 2004; Rolle und Mayr 2007). Unfortunately, high antigenicity and absence of adverse side effects are often incompatible. Only MLV vaccines and vector vaccines are able to activate cytotoxic T cells ($CD8^+$) capable of destroying virus-infected host cells. Inactivated vaccines and subunit vaccines need additional adjuvants, such as saponin or immune stimulating complexes (ISCOMs) to make activation of $CD8^+$ T cells possible. Immune response induced by T helper cells ($CD4^+$) alone, as is usually the case through inactivated vaccines, will not be effective in destroying virus-infected cells, although humoral immune response will be enhanced (Selbitz and Moos 2006).

2.2.2 Canine distemper vaccines

2.2.2.1 Modified live virus vaccines

In MLV vaccines, the virus is rendered avirulent by attenuation but is still able to replicate in the host. The infected cells then process endogenous antigen. In this way, live viruses trigger a response dominated by $CD8^+$ T cells, a Th1 response. Vaccination with a MLV vaccine closely mimics natural infection and stimulates both humoral and cellular immune responses

(Tizard 2004). The majority of CD vaccines currently contain either the avian cell culture adapted Onderstepoort strain or the canine kidney cell adapted Rockborn strain.

All commercially available CD vaccines in Europe are MLV vaccines registered for use in domestic dog, mink (*Mustela vison*) or domestic ferret. These vaccines proved to be safe and efficacious in the species they are developed for, but used in other species residual virulence may cause clinical disease or death. The problem faced when considering vaccination against canine distemper in non-domestic carnivores is the variation between and within species in their reaction to MLV vaccines. As mentioned before, there is a clear difference in vaccine efficacy and adverse effects between the two major vaccine types Onderstepoort and Rockborn. Red pandas, black-footed ferrets, European mink, gray foxes and African wild dogs are highly susceptible to vaccine-induced illness with canine kidney cell adapted MLV vaccines (Table 4). Chicken-cell adapted MLV vaccines specifically attenuated for domestic ferrets seem to be safe and efficacious in maned wolves, bush dogs and fennec foxes (Montali 1983) but caused disease in several species of mink, ferrets, gray foxes and pandas. Therefore, since 1985, safe alternatives such as inactivated virus vaccines, subunit vaccines or recombinant vaccines have been recommended for CD vaccination in non domestic-carnivores (Montali et al. 1994), even though the efficacy of inactivated vaccines against CDV infection has been questioned (Appel et al. 1984; Sikarskie et al. 1991).

Table 4 Examples of vaccine-induced canine distemper. Evidence of CDV was provided by clinical (c) or pathological (p) findings, by virus isolation (v), RT-PCR (pc) or serological methods (s). The vaccines involved were of Onderstepoort type (adapted in avian cells) or Rockborn type (adapted in canine kidney cells). n.r. = not reported.

Family	Species	Scientific name	Vaccine type	Evidence	Reference
Ailuridae	Red panda	*Ailurus fulgens*	Onderstepoort	c, p	Erken et al. 1972
			Rockborn	c, p, v	Bush et al. 1976
			n.r.	c, p	Itakura et al. 1979
Canidae	African wild dog	*Lycaon pictus*	n.r.	c, p, s	Mc Cormick 1983
			Rockborn	c, p, s	Brahm 1984
			Rockborn	c, p,	Durchfeld et al. 1990
	Bush dog	*Speothos venaticus*	Rockborn	c, p	Mc Innes et al. 1992
	Fennec fox	*Fennecus zerda*	n.r.	n.r.	Montali et al. 1987
	Gray fox	*Urocyon cinereoargentus*	Onderstepoort Rockborn	c, p	Halbrooks et al. 1981
			Onderstepoort Rockborn	c, p, s	Scott 1997
	Maned wolf	*Chrysocyon brachyurus*	Rockborn	c, p, s	Thomas-Baker 1985
Mustelidae	Black-footed ferret	*Mustela nigripes*	Onderstepoort	c, p, s	Carpenter et al. 1976

Family	Species	Scientific name	Vaccine type	Evidence	Reference
Mustelidae	Black-footed ferret	*Mustela nigripes*	Rockborn	c, p, s	Pearson 1977
	Domestic ferret	*Mustela putorius furo*	Rockborn mink derived ?	c, p, s	Gill et al. 1988
	European mink	*Mustela lutreola*	Onderstepoort	n.r.	Montali et al. 1994
			Onderstepoort	c, p, v, s	Sutherland-Smith et al. 1997
			Onderstepoort	c, p, s, pc	Ek-Kommonen et al. 2003
Procyonidae	Kinkajou	*Potos flavus*	Rockborn	c, p, s	Kazakos et al. 1981

2.2.2.2 Inactivated vaccine

Inactivated vaccines are based on antigens inactivated by heat or chemicals. In contrast to MLV vaccines, inactivated vaccines act as exogenous antigens. They commonly stimulate responses dominated by $CD4^+$ Th2 cells (humoral immune response), which may not be the most appropriate response, but may be safer (Pastoret et al. 1997; Tizard 2004).

Currently, there is no inactivated CDV vaccine commercially available due to their limited efficacy compared to MLV vaccines in domestic dogs and the absence of a commercially interesting market for non-domestic animals (Appel and Montali 1994). A formalin-inactivated CDV vaccine, containing $Al(OH)_3$ as adjuvant (Matern and Klöppel 1988), was produced in Germany to a limited extent since 1982 (Beringwerke AG, Marburg, Germany and Intervet International GmbH, The Netherlands). This was done out of courtesy to zoos and circuses in Germany in urgent need for a safe CDV vaccine for their CDV-susceptible carnivore species. This vaccine is not registered and not commercially available. Within the scope of a long term trial on its efficacy in zoo carnivores, it was available to zoos on demand based on a special permission according to § 17c Abs. 4 No. 2 of the German law on epizootic disease.

For most non-domestic carnivore species, only few data is available on the efficacy of inactivated vaccines. Although, inactivated CDV vaccines are safe, many immunised animals will develop low or no humoral immunity (Montali et al. 1983; Williams et al. 1996; van Heerderen et al. 2002). On the other hand, Franke et al. (1989) described safety and efficacy of an inactivated CDV vaccine for more than 100 wild species held in captivity.

Vaccination studies with inactivated vaccine have been conducted in African wild dogs (Visee 2001; van de Bildt et al. 2002; van Heerderen et al. 2002; Cirone et al. 2004) with controversial results, possibly due to different adjuvants used. In hybrid ferrets, immunity

produced after vaccination with inactivated CDV vaccine was incomplete and antibody titres were significantly lower than in ferrets vaccinated with MLV vaccines (Williams et al. 1996).

Analysis of data available at the Paul Ehrlich Institute, Germany, demonstrated good results after vaccination with inactivated vaccine in maned wolves, ring-tailed coati (*Nasua nasua*), wolverine, giant otter (*Pteronura brasiliensis*), small-clawed otter, bush dog, African wild dog and red panda (Selbitz and Moos 2006).

2.2.2.3 CDV-ISCOM subunit vaccine

An experimental subunit vaccine incorporating the F and H surface protein of CDV into immuno stimulating complexes (ISCOMs) has been developed and tested in domestic dogs and harbour seals, producing humoral and cellular immunity (de Vries et al. 1988; Visser et al. 1992). ISCOMs are stable complexes containing cholesterol, phospholipids, saponin and antigen, and can be used as an adjuvant. Micelles can be constructed using protein antigens and a matrix of a saponin mixture called Quil A. They are highly effective in targeting antigens to the antigen processing cells while the saponin activates these cells promoting cytokine production and the expression of co-stimulatory molecules. Depending on the antigen and the adjuvant composition, Th1 or Th2 response can be stimulated (Tizard 2004). Although the immunity achieved is not sterile (infection of the upper respiratory tract occurs), CDV-ISCOM vaccinated seals were protected from a potentially lethal challenge with the closely related PDV (Visser 1992). The ISCOM vaccine has been used experimentally in several European zoos (Philippa 2007). CDV-ISCOM vaccination seemed to be safe and efficacious in European mink, Eurasian otter, Asian small-clawed otter, red pandas, maned wolves, and Malay civets (*Viverra tangalunga*), but produced only low neutralising antibody titres in the African wild dog (Philippa 2007).

2.2.2.4 Recombinant vaccines

A canarypox-vectored CDV vaccine is commercially available in the USA for the use in domestic ferrets (Purevax® Ferret Distemper, Merial Limited. Duluth, GA, USA) with high efficacy and safety (Williams and Montali 1998; Wimsatt et al. 2001, 2003). Its extra-label use in all susceptible species in zoos is recommended by the American Association of Zoo Veterinarians (AAZV), although only limited published data on its efficacy in non-domestic species exist (Coke 2005; Philippa 2007). In the EU its use is not permitted as it is a non-registered genetically modified organism (Philippa 2007) although currently several other recombinant vaccines have been registered for domestic species (Moulin 2005). The major problem in developing and registering a recombinant CDV vaccine in the EU would be the enormous registration fees at the European Medicines Agency (EMEA) in London (Selbitz and Moos 2006).

The main advantage of recombinant canarypox vaccines is their safety in mammals. Members of the *Avipox* genus (e.g. fowlpox and canarypox) are non-pathogenic and replication-deficient in mammals due to their natural host range restriction to avian species. However, they still have the ability to enter mammalian cells, reach an early stage of morphogenensis, and express exogenous genes (Sutter and Moss 1992; Somoghi et al.

1993). Protective cellular and humoral immunity is induced in the absence of the complete virus, therefore eliminating the possibility of infection with CDV. Canarypox virus generally appears to be superior to fowl pox virus in the induction of immune response in mammals (Moss 1996).

2.2.2.5 DNA vaccines

DNA vaccines or polynucleotide vaccines contain DNA that encodes foreign antigens. The DNA can be inserted into a bacterial plasmid that acts as a vector. The DNA will be transcribed into mRNA when injected in a host cell and translated into endogenous vaccine protein. The plasmid cannot replicate in mammalian cells. As endogenous antigens, induce neutralising antibodies and activate Tc cells and dendritic cells, DNA vaccines produce a strong Th1 response. The major advantage of the DNA vaccine is its safety because only a particular epitope of the wild-type strain is used and not the whole infectious virus particle (Tizard 2004).

DNA vaccines are still in an experimental state, but so far have shown promising results. A DNA vaccine containing plasmids of the F and H proteins induced persistent humoral immune response in mice and protected them against intracerebral challenge (Sixt et al. 1998). A DNA vaccine containing the N, F and H protein of a virulent CDV strain, developed by Cherrpillod et al. (2000), has proved to be protective against challenge with wild-type CDV.

2.2.3 Vaccine schedules

Vaccination schedules depend on the knowledge of duration of protective neutralising antibody titres raised after vaccination (Greene and Appel 2006). Controlled vaccination studies are limited in non-domestic carnivores. Challenge infections in endangered or threatened carnivores should not be conducted from an ethical point of view and therefore data have to be extrapolated from domestic animals or other related species (Philippa 2007).

Duration of immunity in vaccinated animals is dependent on memory cells and not simply on effector cells producing antibodies. As effector cells are generally short-lived, the memory of the host to an invading organism will not necessarily be recognised through antibody levels, yet immunity can still occur. The usefulness of titres to measure immunity is, therefore, limited to a few disease agents, such as canine distemper, canine parvovirus and adenovirus. However, even here limitations with current technologies, lack of standardisation among laboratories and lack of validation for non-domestic species affect interpretation of results (Gumly 1999; Philippa 2007). Vaccine schedules in otters are derived from schedules of domestic dogs or domestic ferrets. If these schedules are appropriate for the different otter species, is still unclear (Tschirsch 1992).

For CDV vaccination in non-domestic species, monovalent inactivated vaccines, subunit vaccines or recombinant vaccines are generally recommended. Animals with clinical illness should not be vaccinated (Montali 1994). In the event of a viral disease outbreak in an animal

collection, all susceptible species should ideally be vaccinated immediately and boostered 10-14 days later, regardless of age and last time of immunisation (Phillips 1989).

Generally, vaccines for CDV are to be given every 3-4 weeks on at least two occasions between 6 and 16 weeks of age (Greene and Appel 2006). Revaccination is to be done after one year (Greene and Appel 2006). In young ferrets, vaccination at 8 weeks of age and two additional boosters at 3 week intervals are recommended (Quesenberry and Orcutt 2004). For basic protection, the formalin-inactivated CDV vaccine available in Germany is to be given at least two times in 2-3 week intervals and is to be boostered yearly. Small mammals shall get a dose of 1 ml, bigger mammals a dose of 2 ml (Selbitz and Moos 2006). Experimental CDV-ISCOM vaccine is to be applied three times at a three week interval (Visser et al.1989; Philippa 2007).

2.2.4 Failures in vaccination

Despite vaccination, outbreaks of CD continue to occur among vaccinated individuals and populations (Rikula 2008; Blixenkrone-Möller et al. 1993; van de Bildt et al. 2002). There are many reasons why a vaccine may fail to confer protective immunity on an animal (Figure 5).

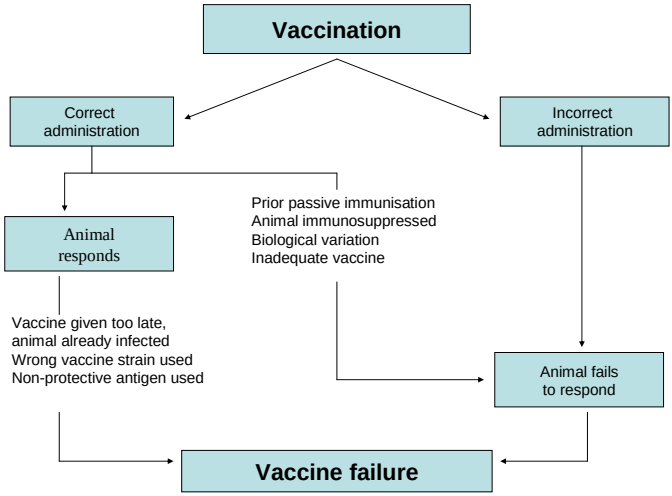


Figure 5 General reasons for vaccine failures (adapted from Tizard 2004).

In many cases, vaccine failure is due to unsatisfactory administration. For example, when using remote delivery systems one must be sure that a full dose is delivered, as syringe darts may rebound quickly on impact and fail to deliver the dose required to elicit a satisfactory

immune response (Aiello 1998). Interruption of the cold chain may be another reason. Lyophilized tissue culture vaccine strains are stable for 16 months under refrigeration (0-4°C), 7 weeks at 20°C and 7 days when exposed to sunlight at 47°C. After reconstitution, a vaccine virus remains stable for 3 day at 4°C and 24 hours at 20°C; however, a reconstituted vaccine should be used within one hour (Greene and Appel 2006).

Unconventional routes of administration or inactivation of live vaccine by use of antibiotics or too much alcohol when swabbing the skin can prevent vaccination success. Another important reason for failure is interference by maternal antibodies. Generally, maternal antibodies decline to insignificant levels by 10-12 weeks but may in extreme cases persist for as long as 16-20 weeks (Tizard 2004). Results from field studies in domestic dogs suggest that even minimal levels of maternal antibodies that are still present at the time of vaccination may impair the ability to respond to vaccination (Rikula 2001).

The use of some drugs, such as tetracycline, chloramphenicol, dapsone, clindamycin, griseofulvin, nalidixic acid and sulphamethoxyparadizine have been associated with inadequate responses to vaccination (Kruth 1998). Concurrent infections at the time of vaccination may stimulate the production of interferon, block the replication of the vaccine virus or be immunosuppressive. Also stress may inhibit the production of an immune response to vaccination (Selbitz and Moos 2006).

Antigens applied simultaneously can interact with each other and with the vaccinated host. These interactions may enhance or reduce the immunogenicity of a particular antigen (Strube 1997). Phillips et al. (1989) demonstrated that MLV CDV and canine adenovirus-1 or canine adenovirus-2 in a multivalent vaccine suppressed lymphocyte responsiveness. Modified life parvovirus antigens in multivalent vaccines have also been suspected to be immunosuppressive (Greene and Appel 2006).

Occasionally, a vaccine may actually be ineffective because production techniques destroyed the protective epitopes or the vaccine contained insufficient amounts of antigen. More commonly, however, an animal may simply fail to mount an immune response. No vaccine can be expected to be 100% effective. Since the immune response is influenced by a great number of genetic and environmental factors, the range of immune responses in a large random population of animals tends to follow a normal distribution. This implies that most animals will produce an average immune response but there will be also a few poor responders as well as excellent responders (Tizard 2004).

3. AIMS OF THE STUDY

The aim of the present thesis was to evaluate and compare the safety and efficacy of CDV vaccines available in Europe in the Eurasian otter and the Asian small-clawed otter, the most common otter species in European zoos and wildlife parks; furthermore to study immune response to an inactivated vaccine in the North American river otter and to compare two methods of CDV antibody determination, an indirect ELISA and the NPLAT. More specifically, the aims were to

1. study the efficacy and safety of an inactivated CDV vaccine in Eurasian otters, North American river otters and Asian small-clawed otters;

2. evaluate the efficacy and safety of CDV-ISCOM vaccine and different MLV CDV vaccines in Eurasian otters and Asian small-clawed otters;

3. examine the immune response in Eurasian otters after booster vaccination with MLV CDV vaccine;

4. explore possible associations of the immune response with age, sex, weight and species;

5. assess differences in immune response according to the vaccine used, and

6. to determine the correlation between an indirect ELISA and the VNT.

4. MATERIALS AND METHODS

Overall 70 otters kept in 23 different zoos and wildlife parks in Germany, the Netherlands (Figure 6), Croatia and Hungary (Figure 7) were included in this study conducted between January 2005 and November 2007.

Figure 6 Participating zoos in Germany and in the Netherlands (●).The numbers on the map refer to the following zoos:(1) Aqua Zoo, Düsseldorf, (2) Artis Zoo, Amsterdam, (3) Tierpark Aschersleben, (4) Wildpark Bad Mergentheim, (5) Braunschweig Zoo, (6) Tierpark Cottbus, (7) Dierenrijk Europa, Eindhoven/Nuenen, The Netherlands, (8) Frankfurt Zoo, (9) Tierpark Görlitz, (10) Hoyerswerda Zoo, (11) Köln Zoo, (12) Wildpark Lüneburger Heide, Hanstedt, (13) Tierpark Neumünster, (14) Tierpark Olderdissen, Bielefeld, (15) Tierpark Osnabrück, (16) Otter-Zentrum, Hankensbüttel, (17) Randers Regenskov Tropical Zoo, Randers, Denmark, (18) Rostock Zoo, (19) Safaripark Beekse Bergen, Hilvarenbeek, The Netherlands, (20) Tierpark Ueckermünde, (21) Zoom Erlebniswelt, Gelsenkirchen.

Figure 7 Participating zoos in Croatia and Hungary (●).The numbers on the map refer to the following zoos: (22) Zagreb Zoo, Croatia, (23) Sóstó Zoo, Sóstófürdo-Nyiregyhaza, Hungary.

4.1 Study animals

Thirty-eight Eurasian otters, 28 Asian small-clawed otters and 4 North American river otters (Figure 8) were vaccinated. The otters were identified by microchips and ranged in age between 8 weeks and 17 years. Every healthy otter in Germany and in the Netherlands previously not vaccinated could have been included in this study. However, many zoos rejected participation, mostly due to problems in catching the otters or due to objections concerning anaesthesia.

Figure 8 North American river otter, Eurasian otter and Asian small-clawed otter, from left to right

Selection of the vaccine type used in the single zoos was influenced by external circumstances. Modified live virus vaccine was only administered in zoos which regularly use this vaccine in otters. In these zoos, the normally used vaccine was applied. In zoos, in which so far otters had not been vaccinated, inactivated CDV vaccine or CDV-ISCOM vaccine was used. Since there were problems in the beginning of the study to get permission to use CDV-ISCOM vaccine in Germany, most of the otters in Germany were vaccinated with inactivated CDV vaccine or MLV vaccine.

Two studies were conducted:

Study 1: Twenty-seven Eurasian otters, 28 Asian small-clawed otters and 4 North American river otters not previously vaccinated against CDV were each vaccinated with one of the vaccines listed in Table 5.

Study 2: Eleven Eurasian otters, regularly vaccinated against CDV, were evaluated for their CDV specific antibody titre one year after their last vaccination. All otters received a booster with MLV vaccine (Nobivac® SHP+LT) and titres after vaccination were determined.

4.2 Vaccines and vaccination intervals

The vaccines used are listed in Table 5. Inactivated CDV vaccine and CDV-ISCOM vaccine were applied three times intramuscularly in the thigh of the animal at 3-4 week intervals. The MLV CDV vaccine was given twice subcutaneously also in a 3-4 week interval. After vaccination, the otters were observed for 30 min for adverse reactions. The animals were monitored daily by the keepers for changes in appetite, growth or any other sign of indisposition or clinical disturbances during feeding times.

Inactivated CDV vaccine and CDV-ISCOM vaccines are experimental vaccines and not approved. CDV-ISCOM vaccine was kindly provided by Prof. T. Kuiken, Erasmus Medical Centre (MC), Rotterdam, The Netherlands. Inactivated CDV vaccine was supplied by courtesy of Bert Geyer, veterinarian of Frankfurt Zoo. For both vaccines, a special permission for its use was needed after § 17c of the German law of epizootic disease which was given by the Paul Ehrlich Institute, Langen, Germany. The MLV vaccines, Nobivac® SHP+LT, Virbagen canis® $SH_{(A2)}$P/LT and Vanguard® 7 are registered for the use in dogs and commercially available. For this study the vaccines were kindly provided by the manufacturers.

Table 5 Canine distemper vaccines used in the study. CDV strains abbreviated as Rockborn (RO), Onderstepoort (OP), Bussell (BU) and Lederle (LE). Minimum titre of CDV per dose (1ml) expressed as $TCID_{50}$ (tissue culture infectious dose 50% endpoint/ml).

Vaccine	CDV strain cell line	Minimum titre of CDV per dose	Other antigens in the vaccine	Adjuvant	Manufacturer
CDV inactivated Batch 000625	RO canine kidney	10^5/ml	none	$Al(OH)_3$	Intervet international GmbH, The Netherlands
CDV-ISCOM Batch 2004-1 16/4/05	BU (OP type) Vero	protein 10µg/ml	none	Quil A	Institute of Virology, Erasmus MC, Rotterdam, The Netherlands
Nobivac® SHP+LT Batch 004320C 74140A	OP type Vero	10^3/ml	ML[1] CAV-2[2] and CPV[3] inact.[4] L[5]+T[6]	no adjuvant	Intervet GmbH, Germany
Virbagen canis® $SH_{(A2)}P$/LT Batch 81112502 1W69	LE (OP type) Vero	10^3/ml	as above	$AL(OH)_3$	Virbac Tierarznei-mittel GmbH, Germany
Vanguard® 7 Batch L40129/130	RO type canine kidney	10^3/ml	ML[1] CAV-2[2] ML[1] CPV[3] inact.[4] L[5]	no adjuvant	Pfizer Pharma GmbH, Germany

[1] modified live
[2] canine adenovirus 2
[3] canine parvovirus
[4] inactivated
[5] *Leptospira canicola* and *Leptospira icterohaemorrhagica*
[6] rabies virus

4.3 Immobilisation and blood-sampling

For blood-sampling, the otters had to be immobilised. In most otters, inhalation anaesthesia was used. Five percent isoflurane in oxygen (5l/min) was fed into an inhalation box. Once the otters were asleep, anaesthesia was continued using a mask with 2-3% isoflurane in oxygen (2l/min, see Figure 9). In rare cases it was impossible to get an otter into the box. In that case a blowpipe was used and ketamine (Ketamin®10%, Essex Tierarzneimittel, München, Germany) and medetomidine (Dormitor®, 1 mg/ml, Pfizer Pharma GmbH, Karlsruhe,

Germany) was applied at a dosage of 5 mg/kg ketamine and 25-50 µg/kg medetomidine. Initial dosage was based on estimated body mass and subsequently on body mass measured during previous anaesthesia.

Figure 9 Inhalation anaesthesia with a portable inhalation anaesthesia machine (Völker Vet, Völker Vet GmbH, Kaltenkirchen, Germany, DE 0217) in an Eurasian otter.

Between 2 and 5 ml blood was taken from the *vena cephalica antebrachii* or *vena jugularis* prior to each vaccination and when possible 12 -14 month after the initial vaccination. To minimise the number of times the animals had to be anaesthetised, no blood was collected after the third vaccination (Table 6). Blood was collected into sterile 5ml EDTA plastic tubes (Kalium EDTA, No. 34.343, Sarstedt, Nürnberg, Germany) and kept at room temperature until processing.

Table 6 Blood sampling and vaccination interval

Study		Days after vaccination			
		day 0	day 21	day 42	day 365
1	Blood sampling	x	x	x	x
	Vaccination	x	x	x	-
2	Blood sampling	x	x	-	x
	Vaccination	x	-	-	-

4.4 Laboratory diagnosis

4.4.1 Processing of blood

Blood was processed within 24 hours after blood sampling. It was centrifuged (Sepatech Heraeus Megafuge 1.0 R, swing-out rotor 2705) 10 minutes at 1350 g, plasma was separated and stored in cryotubes (1.0 ml Eppendorf AG, Hamburg, Germany) in aliquots of 500 µl at -20°C.

4.4.2 Cell culture

For the VNT and for the ELISA Vero cells were used. This cell lineage which was isolated from kidney epithelial cells of African green monkeys (*Cercopithecus aethiops*) (Yasumura and Kawakita 1963) shows adherence and is suitable for morbillivirus research (Shishido et al. 1967).

The cells were cultivated in 175 cm^2 flasks (Nunc GmbH Wiesbaden, Germany) with a total volume of 10 ml at 37°C, 5% CO_2 and 70-80% humidity (gassed incubator BB16 Funktionline, Heraeus, Berlin, Germany). Dulbecco`s Modified Eagle Medium (DMEM, GIBCO®, Invitrogen GmbH, Karlsruhe, Germany) was used, supplemented with 5% foetal calf serum (FCS, Invitrogen GmbH, Karlsruhe, Germany) and 50 µg/ml Gentamicin. Cells were split when reaching confluency, i.e. every three to four days. The medium was then discarded and the cells were washed once with 5 ml DMEM. Subsequently, cell detachment was achieved by incubation with 0.5 ml 0.5% Trypsin-EDTA (GIBCO® 25300, Invitrogen GmbH, Karlsruhe, Germany) at room temperature for 6 minutes. Detached cells were re-suspended in 10 ml DMEM, split 1:4 and 1:10 and provided with fresh medium including the required additives.

4.4.3 Viruses

For serological testing the following virus strains were used:

1. CDV strain Onderstepoort 92 (Haig 1948), kindly supplied by Prof. Volker Moennig, Tierärztliche Hochschule, Hannover, Germany, was used in NPLAT and

2. CDV strain Bussell (Bussell and Karzon 1965), a Vero cell adapted clone of the Onderstepoort strain, was used for the ELISA. Bussell`s strain was kindly provided by Prof. Thijs Kuiken, Institute of Virology, Erasmus MC, Rotterdam, The Netherlands.

4.4.4 Neutralising peroxidase-linked antibody test (NPLAT)

CDV-specific neutralising antibody titres were determined by using the neutralising peroxydase-linked antibody test (NPLAT). The neutralisation protocol was a modified version of that described by Appel and Robson (1973) and has been validated by Zaghawa et al. (1990).

A constant amount of virus was mixed with serial dilutions of the test serum. Vero cells were used for verification of CDV. Onderstepoort 92 CDV strain was applied in the test. A goat anti-mouse-IgG-peroxidase conjugate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used to indirectly visualise the presence of antigen-antibody-complexes in virus-infected cells (Figure 10).

2-log dilution series (1:20–1:2560) of serum samples were tested for their ability to neutralise 100 median tissue culture-infectious doses ($TCID_{50}$) of CDV in Vero cell culture. The required

100 $TCID_{50}$ dose of the virus was gained by dilution of the virus and verified in a control titration. Samples were first screened at a dilution of 1:20. All positive reactors were titrated.

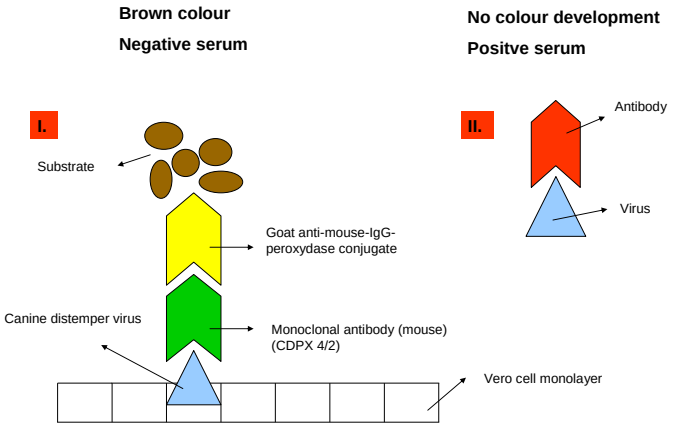


Figure 10 Principle of the NPLAT to detect antibodies against canine distemper virus. **I**. Serum without antibodies. **II.** Positive serum with neutralising antibodies, no binding of virus/antibody-complex to cells.

The NPLAT was carried out as follows: Frozen serum was thawed at room temperature, diluted 1:20 with DMEM, supplemented with 2% penicillin/streptomycin and cold-inactivated (5°C) for 12 hours to be depleted of complement activity. Subsequently, the samples were centrifuged 10 min at 10.000 rpm. Hundred µl serum dilutions (1:20 to 1:2560) were pre-incubated with 50µl virus (100 $TCID_{50}$/50µl) for 1 hour at 37°C in 96 well micro-titre plates (Nunc GmbH, Wiesbaden, Germany) before addition of 50 µl Vero cells (1.8 x 10^5 /ml). Virus/serum mixture was incubated for 3 days in a CO_2 incubator at 37°C. Positive control sera and blank culture medium for cell growth control were set up with every analysis. Positive control sera were obtained from another member of the mustelid family, the stone marten and from the red fox. The infectivity of the virus used was determined. Vero cells served as negative control.

After 3 days of incubation the plates were washed once with 30% PBS (1/3 PBS + 2/3 aqua destillata) and the cells heat-fixed at 80°C for 12 hours. Then, a monoclonal mouse anti-CDV antibody (CDPX 4/2) directed against the P protein (Harder et al. 1991) was applied. Hundred µl of the PBS diluted monoclonal antibody (1:500) was filled in every well and incubated for 2 hours at room temperature. Subsequently, the supernatant was decanted and the wells were washed four times with 300 µl PBS per well. Hundred µl diluted (1:1000 PBS) goat-anti-mouse-IgG peroxydase conjugate was then filled into every well. After two

hours incubation at room temperature, the supernatant was removed and the wells were washed again four times with 300 µl PBS per well. Fifty µl of a substrate stock solution (5 ml natrium-acetate-buffer + 300 µl 3-amino-9-ethyl-carbazole) (AEC stock solution, Sigma®) and 150 µl 30% H_2O_2 was then added into each well. After 20 minutes reaction time the wells were washed with water and air-dried.

Virus neutralising antibody titres were determined microscopically on the basis of brown cell dying and expressed as the reciprocal of the highest dilution that showed no dying of the cells after 3 days incubation. The neutralisation titre was then calculated after the method of Spaermann and Kärber (Spaermann and Kärber 1985) as 50 neutralisation dose (ND_{50}). Every well which showed the slightest sign of brown cells was considered as antibody negative.

4.4.5 Indirect enzyme-linked immunosorbent assay (ELISA)

ELISAs are among the most important immunoassays employed in veterinary medicine (Tizard 2004). They are easy and rapid to perform, conducted in most veterinary diagnostic labs and commercially available as kits. Future evaluation of vaccine-induced antibody titres would be more practical if the ELISA test can be used. Therefore antibody titres were also determined with an indirect ELISA and titres were compared with VNT titres.

The ELISA protocol was a modified version (Philippa 2007) of that described by Örvell et al. (1985). Horseradish-peroxydase (HRP) conjugated Protein A was used to detect the CDV-specific immunoglobulin bound to the antigen coated wells.

The ELISA was carried out in polysterene 96 well micro-titre plates coated with antigen (Bussell`s strain) or Vero cell lysate (background optical densitiy), kindly provided by Prof. Thijs Kuiken, Institute of Virology, Erasmus MC, Rotterdam (Figure 11). The plates were delivered frozen at - 20° Celsius and stayed frozen at that temperature until used.

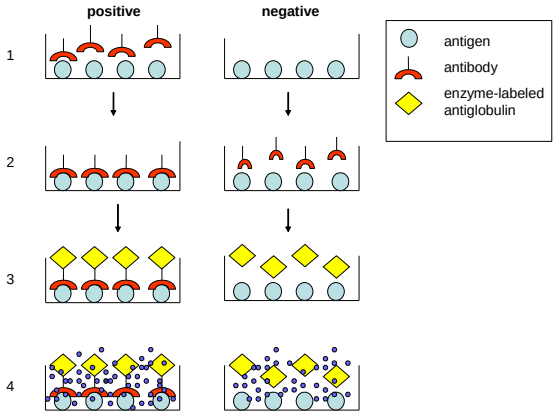


Figure 11 The indirect ELISA technique for antibodies. (1) Antigen coated micro-titre plates are incubated with test sera. (2) Specific antibodies bind to the antigen. (3) Addition of protein A-peroxydase conjugate or enzyme-labeled antiglobulin. (4) Adding of substrate solution and quantification of colour reaction by spectophotometry.

The indirect ELISA was carried out as follows:

Coating CDV ELISA plate: Vero cells were seeded into roller bottles or 162 cm^2 cell culture flasks (Costar plastics, Cambridge, MA) at a concentration of $2x10^5$ cells/ml. DMEM (LONZA, Verviers, Belgium) supplemented with penicillin/streptomycin and 5% FCS (Greiner bio-one) and directly infected with 10^6 $TCID_{50}$ CDV (Bussell`s strain). Five to seven days after infection, when 80-90% of the cells showed cytopathic effect, the cells were harvested and spun down (20 min, 2000 rpm, rotor 10 cm.), and after clarification the pellet was resuspended in PBS (pH 7,4). One percent cell lysis buffer NP-40 (Biosource cat #FNN0021) was added. The cell lysate was titrated with known positive and negative serum samples to achieve a concentration with good response to positive samples and low background interference.

96-well polysterene micro-titre plates (Costar 96 well EIA/RIA plate cat. 3590, Cambridge, MA) were then coated with 100 µl of virus antigen suspension (10^6 $TCID_{50}$ CDV, Bussell`s strain) per well diluted in PBS (pH 7.4). The plates were incubated for 1 hour at 37°C and subsequently washed three times with PBS-Tween 20 (Merck) 0.05%. The extraneous binding sites were blocked with PBS supplemented with 0.5% bovine serum albumine and 0.2% milk powder at 37°C for 30 minutes. Subsequently, the plates were washed three times with PBS-Tween 20 0.05%. Hundred µl ELISA buffer (PBS with 0.2% bovine serum albumine, 0.1% low fat milk powder and 5% NaCl) was then put into each well and the plates frozen at -20°C.

Coating Vero cell ELISA plate - negative control. The procedure of virus production and clarification for the Vero cell plate was done as described above without infecting the Vero cells with virus. After clarification and collecting the Vero cell pellet, a Bradford test was used to match protein concentration with that of the CDV plate coating. Subsequently 96 well micro-titre plates were coated with 100 µl Vero cell lysate with the same protein concentration as de CDV coating.

Indirect ELISA. Frozen serum samples and micro titre plates were thawed at room temperature. 2-log dilution series of pre-diluted serum samples were made (1:20 to 1:2560) using a buffer consisting of PBS with 0.2% bovine serum albumine (Albumine Sigma®, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 0.1% milk powder (Magermilchpulver Sucofin, TSI GmbH&Co, Lever) and 5% NaCl. Hundred µl of each dilution was added (in duplicate) to the wells. After incubation for 1 hour at 37°C, the plates were washed three times (Automated Strip Washer, Bio-Tek® Elx50™, Bad Friedrichshall, Germany) with buffer containing 0.05% Tween 20 (Serva Feinbiochemica, Heidelberg, Germany). Hundred µl horseradish-peroxydase conjugated protein A (Zymed Laboratories, Invitrogen, 10-1023, 2 ml, Lot No 60606041) in a dilution of 1: 12000 was added to each well and the plates were incubated again for 1 hour at 37°C. The plates were washed three times with buffer containing 0.05 % Tween 20. After the addition of 100 µl tetramethylbenzedine solution (TMB Southern Biotech, Birmingham, Alabama, USA) to each well and the development of colour, the reaction was stopped by the addition of 50 µl sulphuric acid solution (1M) per well. The resulting optical density (OD) was read in a computer-assisted ELISA reader (Tecan Sunrise, Software Magellan, Tecan Deutschland GmbH, Crailsheim, Germany) at 450 nm. A negative and positive control was used in each test. An ELISA using uninfected Vero cell lysate was used as a negative control for each sample tested, providing the background optical density. Every analysis was conducted with two 96 well micro titre plates. One was coated with antigen the other was coated with VERO cell lysate. Both plates were handled exactly the same. Control sera from known positive animals were included in the test. An OD of three times the background OD (Vero cell coated plate) was considered positive. The ELISA titre was expressed as the reciprocal of the maximum dilution showing positive absorbance.

4.4.6 Expressing and presenting serological results

Serological results are recorded as the reciprocal of the endpoint dilution. Results are presented graphically as single case depictions and as geometric mean titres (GMT) with 95% confidence interval (CI). Seronegativity and seropositivity were defined by the absence or presence of detectable VN CDV specific antibodies at a 1:20 dilution of serum. At a 1:10 dilution most otter sera were toxic to Vero cell cultures. Therefore sera were diluted 1:20 and titres <20 were regarded as negative. To simplify statistical analysis, undetectable titres (< 20) were set at 10. Based on published data in domestic ferrets, domestic dogs and several other carnivore species, in which protective titres ranged between 20 and 100 (Appel 1969; Montali et al. 1983; Wimsatt et al. 20031; Wimsatt et al. 2003, Greene and Appel 2006), titre values greater or equal 80 were regarded as high and protective, titres between 40 and 80 as medium and questionably protective and titres between 20 and 40 as low and unlikely to

be protective (Table 7). Animals with VN titres ≥20 prior to vaccination were excluded from calculating mean titres.

Table 7 Definition of serological results.

Titre	Intensity	Presumed effect
≥80	high	protective
40-79	medium	questionably protective
20-39	low	not protective
<20	not measurable	negative

4.5 Statistical analysis

Statistical analyses were performed using SPSS for Windows version 13.0. Statistics are given as geometric mean titres (GMT) with 95% confidence interval. *P* values are for two-tailed tests. For all statistical tests, $P \leq 0.05$ was considered significant.

Fisher`s exact test (Scherrer 1984) was used to compare the number of otters with titres ≥1:20 and ≤1:20 (percentage of seroconversion) after vaccination with different vaccines in different otter species, and to compare the percentage of protective titres (≥80) after vaccination. One-way repeated measures analysis and two-way ANOVA were used to assess titre data.

A general linear model was applied to investigate the influence of species, age, body mass and sex on the immune response as expressed by VN titre after two vaccinations to CDV-ISCOM vaccine. The four age groups of otters were: (1) cubs = ≤ 12 weeks, (2) juvenile = ≤ 1 year, (3) subadult = < 3 years, (4) adult = 3 years and more. Residuals were examined using normal probability plots and tested for normality with the Lillifors test (Wilkinson 1999).

To investigate the relationship between ELISA and VN antibody titres, a linear regression was conducted. The relationship was given by the equation

$$y=a*x+b$$

where y is the VN titre and x the ELISA titre, b is the y intercept and a the slope of the regression line.

5. RESULTS

None of the 70 vaccinated otters showed clinical signs of CD infection, and no local or systemic side effects that could be attributed to vaccination were noticed. The death of five Eurasian otters (LL24, LL25, LL28, LL32, LL33), one North American river otter (Lc1) and one Asian small-clawed otter (Ac14) before the end of the study seemed not to be related to CDV. Three Eurasian otters (LL24, LL25, LL28) were tested negative for CDV by IF test and post-mortem examinations and due to observations made, regarding point of time and circumstances, the death of the other otters seem not to be associated to CDV vaccination or CDV (Appendix II).

Study 1: Otter previously not vaccinated

5.1 Inactivated CDV vaccine

5.1.1 Sample distribution

Twelve Eurasian otters, 12 Asian small-clawed otters and four North American river otters previously not vaccinated and from ten different zoos (Table 8) were vaccinated with inactivated CDV vaccine.

Table 8 Species, number, and location of previously unvaccinated otters vaccinated with inactivated CDV vaccine.

Species	n	Animal ID	Zoo
Eurasian otter	4	LL1/LL2/LL3/LL4	Tierpark Osnabrück
	3	LL7/LL8/LL9	Rostock Zoo
	5	LL10/LL11/LL12/LL13/LL14	Tierpark Hoyerswerda/Neumünster
North American river otter	2	Lc1/Lc2	Wildpark Bad Mergentheim
	2	Lc3/Lc4	Zoom Erlebniswelt Gelsenkirchen
Asian small-clawed otter	4	Ac1/Ac2/Ac3/Ac4	Aqua Zoo Düsseldorf
	4	Ac5/Ac6/Ac7/Ac8	Tierpark Cottbus
	2	Ac9/Ac10	Frankfurt Zoo
	2	Ac11/Ac12	Tierpark Aschersleben

Sex ratio and age-class composition of the otters are shown in Table 9.

Table 9 Sex ratio and age-class composition in otters vaccinated with inactivated CDV vaccine

Species	Sex	n	Age-class			
			cub	juvenile	subadult	adult
Eurasian otter	m	7	-	3	2	2
	f	5	-	2	-	3
Asian small clawed otter	m	5	1	1	-	3
	f	7	1	2	-	4
North American river otter	m	1	-	-	-	1
	f	3	-	-	2	1

5.1.2 Prevalence of CDV-specific antibodies

Of 28 otters, all reported as previously unvaccinated against CDV, 18 (64%) were sero-negative for CDV-specific antibodies tested by VNT and ELISA. Ten otters (36%) were sero-positive by ELISA and/or VNT. Low antibody titres (≤ 40) were found in one Eurasian otter and five Asian small-clawed otters. High antibody titres (≥ 80) were detectable in two Eurasian otters and one North American river otter (Table 10).

5.1.3 Sero-response after vaccination with inactivated CDV vaccine

5.1.3.1 Eurasian otter

No seroconversion was observed after two vaccinations in any Eurasian otter sero-negative at day 0 (9/12). All responding otters (3/12) revealed CDV-specific antibodies prior to immunisation. These individuals showed an increase in antibody titres (Figure 12).

Pre-vaccination titres in Eurasian otters (Table 10) ranged from 33 to 160 (GMT VN: 84; GMT ELISA: 50). After the first vaccination, high antibody titres were detected in all otters measured by VNT and ELISA (GMT VN: 83; GMT ELISA: 160). Antibody titres increased further after the second vaccination (GMT VN: 376; GMT ELISA: 403). One year after initial vaccinations, titres had declined but remained still high in two of the three responding Eurasian otters (GMT VN: 84; GMT ELISA: 40).

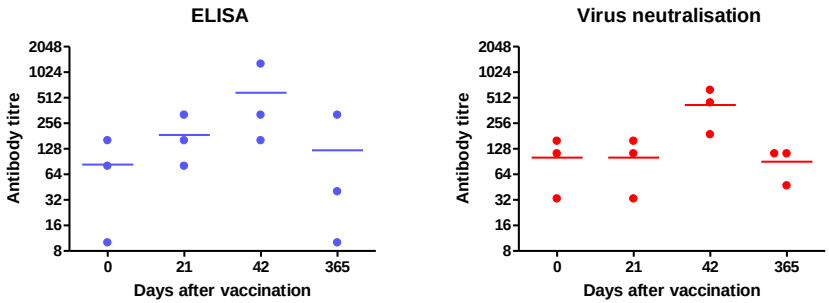


Figure 12 Serum antibody titres measured by ELISA and VNT following vaccination with inactivated CDV vaccine in Eurasian otters that were sero-positive prior to vaccination (n=3). Single-case depiction. Dots indicate antibody titres measured by ELISA and VNT before the 1st vaccination (day 0), at times of the 2nd vaccination (day 21), 3-4 weeks after the 2nd vaccination (day 42) and one year after the 1st vaccination (day 365). The horizontal line depicts the mean. All Eurasian otters responding to vaccination had detectable pre-vaccination titres.

Table 10 Titre values measured by ELISA and VNT in Eurasian otters (n=12) before and after vaccination with inactivated CDV vaccine. Blood was collected prior to the 1st vaccination (day 0), at times of the 2nd vaccination (day 21), 3-4 weeks after the 2nd vaccination (day 42) and one year after the 1st vaccination (day 365). Titre values <20 were not measurable and were set as 10.

Animal ID	Antibody titre ELISA				Antibody titre VNT			
	day 0	day 21	day 42	day 365	day 0	day 21	day 42	day 365
LL1	10	10	10	10	10	10	10	10
LL2	10	10	10	10	10	10	10	10
LL3	10	10	10	10	10	10	10	10
LL4	10	10	10	10	10	10	10	10
LL7	10	10	10	10	10	10	10	10
LL8	10	80	160	40	33	33	188	47
LL9	80	160	1280	320	112	112	447	112
LL10	10	10	10	10	10	10	10	10
LL11	160	320	320	10	158	158	630	112
LL12	10	10	10	10	10	56	10	10
LL13	10	10	10	10	10	10	10	10
LL14	10	10	10	10	10	10	10	10

5.1.3.2 North American river otter

No seroconversion was observed after two vaccinations in any North American river otter (n=3) sero-negative at day 0. Only the one North American river otter sero-positive prior to vaccination showed an increase in antibody titre after vaccination (Table 11).

Table 11 Titre values measured by ELISA and VNT in North American river otters after vaccination with inactivated CDV vaccine. Blood was collected prior to the 1st vaccination (day 0), at the time of the 2nd vaccination (day 21), 3-4 weeks after the 2nd vaccination (day 42) and one year after the 1st vaccination (day 365). Undetectable titres were set as 10.

Animal ID	Antibody titre ELISA				Antibody titre VNT			
	day 0	day 21	day 42	day 365	day 0	day 21	day 42	day 365
Lc1	40	160	160	n.d.	80	112	224	n.d.
Lc2	10	10	10	n.d.	10	10	10	n.d.
Lc3	10	10	10	10	10	10	10	10
Lc4	10	10	10	10	10	10	10	10

n.d. = not done

5.1.3.3 Asian small-clawed otters

Seventy-five percent (9/12) of the vaccinated Asian small-clawed otters were sero-negative measured by VNT at day 0 (Table 12). In three of these otters antibody titres ranging from 20-80 were detectable when measured by ELISA. No sero-conversion was observed after two vaccinations with inactivated CDV vaccine in 89% (8/9) of the otters. One otter showed low VN antibody titres (20) after the first and second vaccination (GMT: 11). When measured by ELISA, an increase in antibody titres was observed in 33% (3/9) of the otters after the first vaccination (GMT ELISA: 18) and in 44% (4/9) after the second vaccination, respectively (GMT ELISA: 23). No CDV-specific serum antibody titre was detectable one year after the initial three vaccinations (Figure 13).

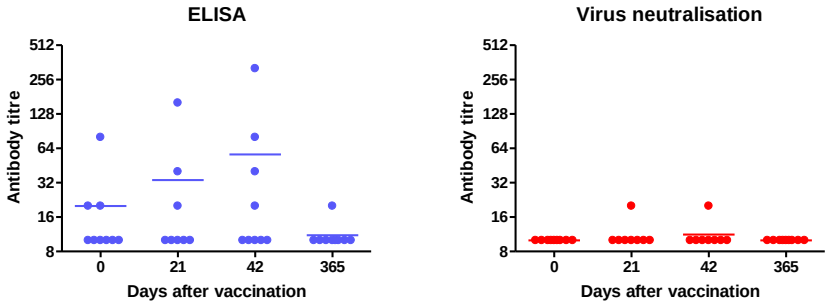


Figure 13 Serum antibody titres measured by ELISA and VNT following vaccination with inactivated CDV vaccine in Asian small-clawed otters (n=9). Single-case depiction: Dots indicate antibody titres measured by ELISA and VNT before the 1st vaccination (day 0), at times of the 2nd vaccination (day 21), 3-4 weeks after the 2nd vaccination (day 42) and one year after the 1st vaccination. The horizontal line depicts the mean.

Table 12 Titre values measured by ELISA and VNT in Asian small-clawed otters that were sero-negative prior to vaccination (n=9) after vaccination with inactivated CDV vaccine. Blood was collected prior to the 1st vaccination (day 0), at the time of the 2nd vaccination (day 21), 3-4 weeks after the 2nd vaccination (day 42) and one year after the initial vaccinations (day 365). Undetectable titres were set as 10.

Animal ID	Antibody titre ELISA				Antibody titre VNT			
	day 0	day 21	day 42	day 365	day 0	day 21	day 42	day 365
Ac1	10	10	10	20	10	10	10	10
Ac2	10	10	10	10	10	10	10	10
Ac3	10	10	10	10	10	10	10	10
Ac5	10	20	20	10	10	20	20	10
Ac7	20	n.d.	40	10	10	n.d	10	10
Ac8	80	160	320	410	10	10	10	10
Ac9	20	10	10	10	10	10	10	10
Ac10	10	10	10	10	10	10	10	10
Ac12	10	40	80	10	10	10	80	10

n.d. = not done

Twenty-five percent (3/12) of the Asian small-clawed otters were sero-positive prior to vaccination (Table 13). Virus neutralising antibody titres ranged from 20-28 (GMT: 25). Sero-response after vaccination was observed in 83% (1/3) of the otters sero-positive prior to vaccination. After the first vaccination, low VN antibody titres were observed (GMT: 17) and stayed low in most cases after the second vaccination (GMT: 18). When measured by ELISA,

antibody titres were medium to high (GMT: 48) and increased further after the second vaccination. No CDV-specific antibody titres were detectable one year after the initial three vaccinations (Figure 14).

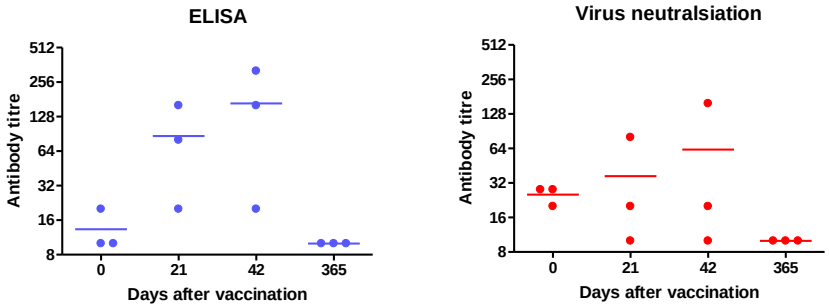


Figure 14 Sero-response in Asian small-clawed otters that were sero-positive prior to vaccination (n=3) after vaccination with inactivated CDV vaccine. Single-case depiction. Dots indicate antibody titres measured by ELISA and VNT before the 1st vaccination (day 0), prior to the 2nd vaccination (day 21), 3-4 weeks after the 2nd vaccination (day 42) and one year after the 1st vaccination. The horizontal line depicts the mean.

Table 13 Titre values measured in sero-positive Asian small-clawed otters (n=3) after vaccination with CDV inactivated vaccine. Blood was collected prior to 1st vaccination (day 0), at the time of second vaccination (day 21), 3-4 weeks after second vaccination (day 42) and one year after the 1st vaccination (day 365). Un-detectable titres were set as 10.

Animal ID	Antibody titre ELISA				Antibody titre VNT			
	day 0	day 21	day 42	day 365	day 0	day 21	day 42	day 365
Ac3	20	160	320	10	28	80	158	10
Ac6	10	80	160	10	20	20	20	10
Ac11	10	10	10	10	10	10	10	10

5.1.4 Comparison of sero-response to inactivated CDV vaccine in the three otter species

Humoral immune response to inactivated CDV vaccine was generally weak. All of the Eurasian otters (9/9) and North American river otters (3/3) sero-negative prior to vaccination as well as 89% (1/8) of the Asian small-clawed otters sero-negative prior to vaccination did not seroconvert after two vaccinations with inactivated CDV vaccine. In none of the otters, CDV-specific antibodies could be observed after one year. No difference was found in the proportion of seroconversion between Eurasian otters and Asian small-clawed otters (Fisher`s exact test, n= 18, P=1.0).

5.2 CDV-ISCOM vaccine

5.2.1 Sample distribution

Four Eurasian otters and 10 Asian small-clawed otters previously not vaccinated from five different zoos (Table 14) were vaccinated with CDV-ISCOM vaccine.

Table 14 Species, number, and location of previously unvaccinated otters vaccinated with CDV-ISCOM vaccine.

Species	n	Animal ID	Zoo
Eurasian otter	2	LL15/LL16	Tierpark Bielefeld
	2	LL35/LL36	Dierenrijk Europa
Asian small-clawed otter	4	Ac13/Ac14/Ac15/Ac16	Artis Amsterdam
	4	Ac20/Ac21/Ac22/Ac23	Safaripark Beekse Bergen
	2	Ac24/Ac25	Frankfurt Zoo

Sex ratio and age-class composition of the otters are shown in Table 15.

Table 15 Sex-ratio and age-class composition in otters vaccinated with CDV-ISCOM vaccine

Species	Sex	n	Age-class			
			cub	juvenile	subadult	adult
Eurasian otter	m	1	-	-	-	1
	f	3	-	-	2	1
Asian small clawed otter	m	4	2	-	1	1
	f	6	2	-	2	2

5.2.2 Prevalence of CDV-specific antibodies

All Asian small-clawed otters (10/10) as well as the Eurasian otters (4/4) were sero-negative for VN CDV-specific antibodies. Twenty percent (2/10) of the Asian small-clawed otters and 50% of the Eurasian otters (2/4) showed low antibody titres (20) when tested by ELISA (Table 16) prior to the first vaccination.

5.2.3 Sero-response after vaccination with CDV-ISCOM vaccine

5.2.3.1 Eurasian otters

After the first dose of CDV-ISCOM vaccine low GMTs were induced as measured by VNT and ELISA (GMT VN and ELISA: 28). Seventy-five percent (3/4) of the otters had detectable VN antibody titres after the second vaccination (GMT VN: 44). High antibody titres were detected after the second dose by ELISA (GMT: 381). One year after the initial vaccinations titres were still high in two otters (50%) as measured by both VN and ELISA (Figure 15, Table 16)

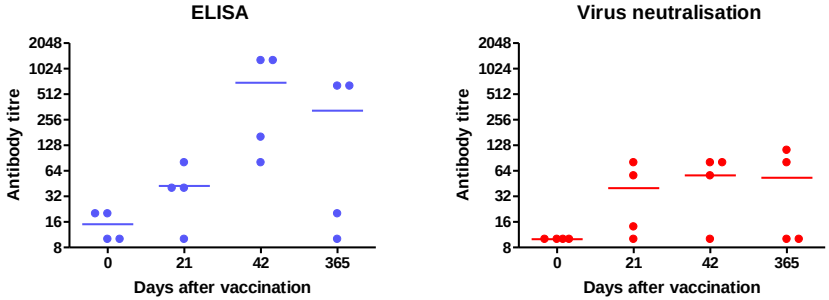


Figure 15 Immune response in Eurasian otters (n=4) after vaccination with CDV-ISCOM vaccine. Single-case depiction. Dots indicate antibody titres measured by ELISA and virus neutralisation before the 1st vaccination (day 0), prior to the 2nd vaccination (day 21), 3-4 weeks after the 2nd vaccination (day 42) and one year after the initial vaccination (day 365). The horizontal line depicts the mean.

Table 16 Titre values measured in Eurasian otters (n=4) measured by ELISA and VNT before and after vaccination with CDV-ISCOM vaccine. Blood was collected prior to the 1st vaccination (day 0), at times of the 2nd vaccination (day 21), 3-4 weeks after the 2nd vaccination (day 42) and one year after the 1st vaccination (day 365). Titre values <20 were not measurable and were set to 10.

Animal ID	Antibody titre ELISA				Antibody titre VNT			
	day 0	day 21	day 42	day 365	day 0	day 21	day 42	day 365
LL15	10	10	160	10	10	10	10	10
LL16	10	40	80	20	10	14	56	10
LL35	20	80	1280	640	10	56	80	80
LL36	20	40	1280	640	10	80	80	112

5.2.3.2 Asian small-clawed otters

Antibody titres between 20 and 640 (GMT: 57) were induced by the first dose of CDV-ISCOM vaccine in Asian small-clawed otters as measured by ELISA. High antibody titres (GMT: 172) were observed after two doses of CDV-ISCOM vaccine (GMT ELISA: 172). Virus neutralising antibody titres were much lower. Only one out of the 10 otters (10%) seroconverted to a low antibody titre (20) after a single dose of the vaccine (GMT: 11). After 2 doses of CDV-ISCOM vaccine, 50% (5/10) of the otters showed seroconversion for VN antibodies (GMT: 18). One year after the initial vaccination 56% (6/9) of the animals showed VN antibody titres ranging from 20 to112 when measured by VN (GMT: 32). Detectable titres measured by ELISA were observed in 77% (7/9) after one year and ranged between 20 and 1280 (GMT: 86) (Figure 16 and Table 17)

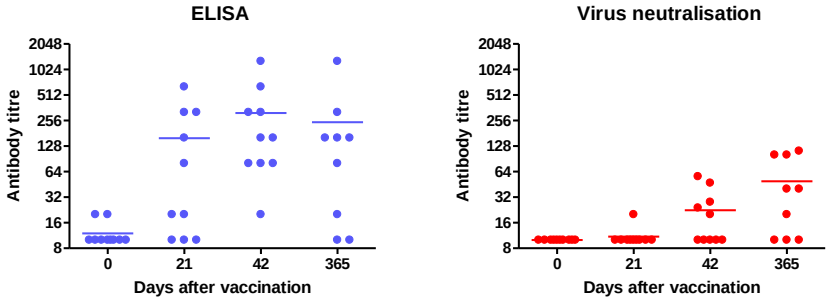


Figure 16 Sero-response in Asian small-clawed otters (n=10) after vaccination with CDV-ISCOM vaccine. Dots indicate antibody titres measured by ELISA and VNT before the 1st vaccination (day 0), prior to the 2nd vaccination (day 21), 3-4 weeks after the 2nd vaccination (day 42) and after one year one (day 365). The horizontal line depicts the mean.

Table 17 Titre values measured in Asian small-clawed otters (n=10) after vaccination with CDV ISCOM vaccine. Blood was collected prior to the 1st vaccination (day 0), at the time of the 2nd vaccination (day 21), 3-4 weeks after the 2nd vaccination (day 42) and one year after the 1st vaccination (day 365). Titre values < 20 were not measurable and were set as 10.

Animal ID	Antibody titre ELISA				Antibody titre VNT			
	day 0	day 21	day 42	day 365	day 0	day 21	day 42	day 365
Ac13	10	20	160	160	10	10	10	40
Ac14	10	10	80	n.d.	10	10	10	n.d.
Ac15	10	10	80	160	10	10	10	40
Ac16	10	10	80	320	10	10	56	112
Ac20	10	20	20	10	10	10	28	101
Ac21	10	160	160	20	10	20	47	101
Ac22	10	80	320	10	10	10	10	10
Ac23	10	320	320	80	10	10	10	10
Ac24	10	640	640	160	10	10	24	10
Ac25	20	320	1280	1280	10	10	24	10

n.d. = not done

5.2.3.3 Comparison of immune response to CDV-ISCOM vaccine in the two otter species

A one-way repeated measures analysis was conducted to compare the reaction to CDV-ISCOM vaccine between Eurasian otters and Asian small-clawed otters. Both otter species showed sero-conversion with significantly increasing VN GMTs ($F_{3,32}$=6.777, P=0.001) after the first (P=0.005) and second (P=0.024) vaccination, which did not differ between the two species (P=0.208). However, a non-significant trend (P=0.085) for VN GMTs being higher in Eurasian otters (GMT: 28 and 44) than in Asian small-clawed otters (GMT: 11 and 16) was noticed. One year post vaccination VN GMTs had decreased in Eurasian otters (GMT: 31) and increased in Asian small-clawed otters (GMT: 32).

Neither the rate of seroconversion (titre ≥20) for VN antibodies nor the percentage of otters with protective titres (VN antibody titre ≥80) did differ significantly between Eurasian otters and Asian small-clawed otters (Table 18). However, a remarkable difference was noticed between titre values measured by ELISA compared to titre values measured by VNT. This was especially pronounced in Asian small-clawed otters. None of the Asian small-clawed otters but 50% (2/4) of the Eurasian otters showed protective titres (VN antibody ≥80) after two doses of CDV-ISCOM vaccine. In contrast, titre values measured by ELISA ranged between 80 and 1280 after two vaccinations. One year after the initial vaccinations titre values measured by ELISA had declined (Eurasian otter GMT: 95 and Asian small-clawed otter GMT: 86, respectively) but were still high (Figure 17 and Table 18).

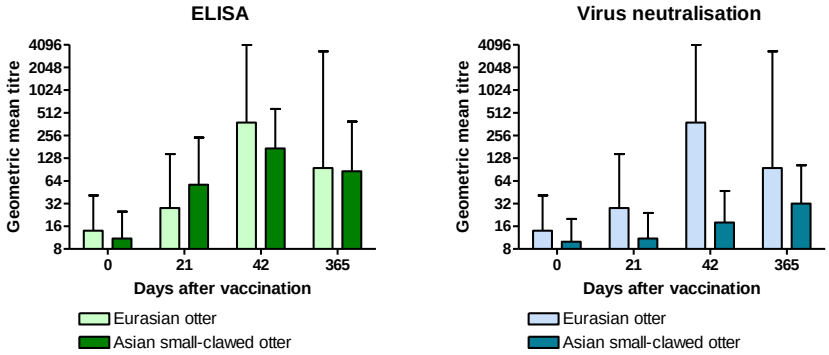


Figure 17 Geometric mean titres in Eurasian otters (n=4) and Asian small-clawed otters (n=10) measured by ELISA and VNT before vaccination (day 0), after the 1st (day 21) and the 2nd (day 42) vaccination and one year after the 1st vaccination (day 365) with CDV-ISCOM vaccine. Note the difference between ELISA and virus neutralisation antibody titres. Error bars depict 95% confidence interval. Undetectable antibody titres were set as 10.

Table 18 Humoral immune response to vaccination with CDV-ISCOM vaccine as measured by ELISA and VNT, shown as VN geometric mean titres (GMT) with 95% confidence interval (CI), percentage of animals with VN antibody titres equal to or greater than 20 (n% ≥20) and percentage of animals with a titre equal to or greater than 80 (n% ≥80) after the 1st and 2nd vaccination and one year post vaccination. *P* = probability.

Species	**Titres pre-vaccination**				**Response after 1st vaccination**				**Response after 2nd vaccination**				**Titres one year post vaccination**			
	n	GMT ELISA (95%CI)	GMT VN (95%CI)	n% ≥20	GMT ELISA (95%CI)	GMT VN (95%CI)	n% ≥20	n% ≥80	GMT ELISA (95%CI)	GMT VN (95%CI)	n% ≥20	n% ≥80	GMT ELISA (95%CI)	GMT VN (95%CI)	n% ≥20	n ≥80
Eurasian Otter	4	14 (7-27)	10	**0**	28 (7-117)	28 (6-143)	50	25	381 (39-3696)	44 (9-212)	75	50	95 (3-3249)	31 (4-245)	50	50
Asian small-clawed otter	10	11 (9-14)	10	0	57 (9-14)	11 (9-13)	10	0	172 (73-404)	18 (11-29)	50	0	86 (24-305)	32 (15-71)	55	33
P						0.085	0.176	0.286		0.085	0.580	0.066		0.106	1.000	1.000

5.3. Modified live virus CDV vaccines

5.3.1 Sample distribution

Eleven Eurasian otters and 6 Asian small-clawed otters not vaccinated previously and originating from seven different zoos (Table 19) have been vaccinated with commercially available MLV vaccines. Three different MLV vaccines were used: Vangard®7, Nobivac® SHP+LT, and Virbagen canis® $SH_{(A2)}$P/LT, which are described in detail in Table 5.

Table 19 Species, number, and location of previously unvaccinated otters vaccinated with MLV CDV vaccine.

Species	Vaccine	Animal ID	Zoo
Eurasian otter	Vangard® 7	LL5	Köln Zoo
	"	LL29/LL30	Tierpark Neumünster
	"	LL32/LL33	Wildpark Lüneburger Heide
	"	L38	Tierpark Neumünster
	Virbagen canis®$SH_{(A2)}$P/LT	LL34	Tierpark Neumünster
	Nobivac®SHP+LT	LL21	Otter Zentrum Hankensbüttel
	"	LL31	Tierpark Görlitz
	"	LL37/LL39	Tierpark Neumünster
Asian small- clawed otter	Virbagen canis® $SH_{(A2)}$P/LT	Ac17/Ac18/Ac19	Tierpark Neumünster
	"	Ac26	Dierenrijk Europa
	"	Ac27	Braunschweig Zoo
	Vangard ®7	Ac28	Dierenrijk Europa

Sex ratio and age-class composition of the otters are shown in Table 20.

Table 20 Sex-ratio and age-class composition in otters vaccinated with MLV CDV vaccine.

Species	Vaccine	n	Sex	Ageclass			
				cub	juvenile	subadult	adult
Eurasian otter	Vangard®7	6	♂	-	1	-	1
			♀	3	-	-	1
	Nobivac®SHP+LT	4	♂	1	-	2	-
			♀	1	-	-	-
	Virbagen canis ®$SH_{(A2)}$P/LT	1	♂	-	-	-	1
			♀	-	-	-	-
Asian small-clawed otter	Vangard®7	1	♂	-	1	-	-
			♀	-	-	-	-
	Virbagen canis ®$SH_{(A2)}$P/LT	5	♂	-	2	-	-
			♀	-	1	2	-

5.3.2 Prevalence of CDV-specific antibodies

All Eurasian otters were sero-negative for CDV-specific antibodies as measured by ELISA. High VN antibody titres were detected in 18% (2/11) of the otters (Table 21). All Asian small-clawed otters were sero-negative for VN CDV-specific antibodies. When measured by ELISA, low VN antibody titres (≤ 40) were observed in 33% (2/6) of the otters (Table 22).

5.3.3 Sero-response after vaccination with modified live virus vaccine

5.3.3.1 Eurasian otters

After the first vaccination, 56% (5/9) of the otters showed sero-conversion (titres ≥20) for VN antibodies (GMT: 66). Twenty-two percent (2/9) reacted positive when measured by ELISA (GMT: 23). High VN antibody titres and antibody titres measured by ELISA, ranging from 160 to 2560, were observed after the second vaccination in 67% (6/9) of the otters. Low to medium antibody titres (20-47) were measured in 33% (3/9) of the individuals. Two of the otters (22%) did not sero-convert until day 42 post vaccination (GMT VN: 206; GMT ELISA: 137). One year after the initial vaccinations, antibody titres could be determined in four otters (45%). High VN antibody titres (224 and 316, respectively) and ELISA titres (320 and 320, respectively) were detectable in two otters (50%) whereas the other two otters showed no or low antibody titres, respectively, as measured by virus neutralisation (GMT: 52) and ELISA (GMT: 57) (Figure 18, Table 21).

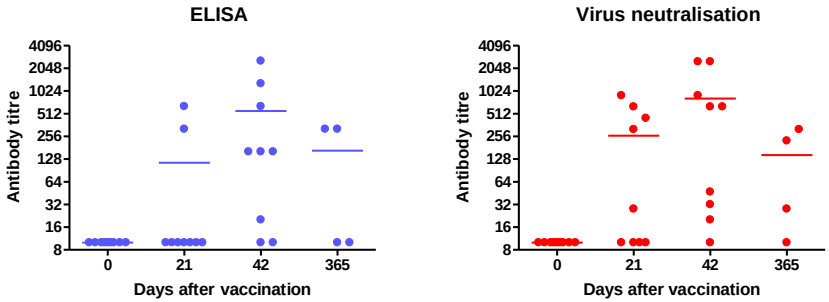


Figure 18 Immune response in Eurasian otters (n=9) after vaccination with MLV CDV vaccine. Single-case depiction. Dots indicate antibody titres measured by ELISA and VNT before the 1st vaccination (day 0), prior to the 2nd vaccination (day 21), 3-4 weeks after the 2nd vaccination (day 42) and one year after the 1st vaccination (day 365). The horizontal line depicts the mean. Un-detectable titres were set as 10.

Table 21 Titre values measured in Eurasian otters (n=11) before and after vaccination with different MLV vaccines. Blood was collected prior to 1st vaccination (day 0), at the time of second vaccination (day 21), three-four weeks after second vaccination (day 42) and one year after the 1st vaccination (day 365). Titre values < 20 were not measurable and were set as 10.

Animal ID	Vaccine	Antibody titre ELISA				Antibody titre VNT			
		day 0	day 21	day 42	day 365	day 0	day 21	day 42	day 365
LL21*	Nobivac®	10	80	320	320	80	188	224	365
LL31	SHP+LT	10	10	1280	320	10	447	2511	224
LL37	"	10	640	2560	n.d.	10	891	2511	n.d.
LL39	"	10	10	160	320	10	10	891	316
LL34	Virbagen canis® $SH_{(A2)}P/LT$	10	10	160	n.d	10	36	631	n.d
LL5*	Vangard®7	10	10	10	10	80	10	10	10
LL29	"	10	10	10	10	10	10	32	10
LL30	"	10	10	160	10	10	28	47	28
LL32	"	10	10	10	n.d.	10	10	10	n.d.
LL33	"	10	320	640	n.d.	10	631	631	n.d.
LL38	"	10	10	20	n.d.	10	10	20	n.d.

n.d. = not done

* = excluded from calculating mean titres

One of the two otters sero-positive prior to vaccination (LL5) had no detectable antibody titre at day 21 and day 42 post vaccination. In the other otter (LL21), a high VN antibody titre was raised, which remained still high one year after the two vaccinations (Table 21).

5.3.3.2 Asian small-clawed otters

All Asian small-clawed otters (6/6) showed high VN CDV-specific antibody titres (GMT: 911) and high antibody titres as measured by ELISA (GMT: 280) after the first vaccination, which increased to very high titres after the second vaccination (GMT VN: 2014; GMT ELISA: 1024). Titres raised after the first vaccine application ranged from 80 to 1258 and from 640 to 3548 after the second dose of the vaccine (Figure 19 and Table 22).

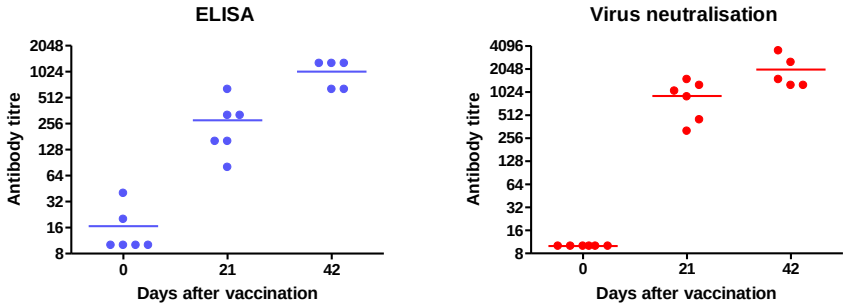


Figure 19 Immune response in Asian small-clawed otters (n=6) after vaccination with MLV CDV vaccine. Single-case depiction. Dots indicate titres measured by ELISA and VNT before 1st vaccination (day 0), prior to 2nd vaccination (day 21), and 3-4 weeks after the 2nd vaccination (day 42). Note the high virus neutralising titres raised after the first vaccination. The horizontal line depicts the mean.

Table 22 Titre values measured in Asian small-clawed otters (n=6) before and after vaccination with different MLVCDV vaccines. Blood was collected prior to 1st vaccination (day 0), at the time of 2nd (day 21) and 3-4 weeks after the second vaccination (day 42). Titre values < 20 were not measurable and are depicted as 10.

Animal ID	Vaccine	Antibody titre ELISA			Antibody titre VNT		
		day 0	day 21	day 42	day 0	day 21	day 42
Ac17	Virbagen canis®	10	640	1280	10	1496	1496
Ac18	SHP/LT	10	80	640	10	1059	2511
Ac19	"	10	160	1240	10	1258	3548
Ac27	"	20	160	n.d.	10	316	n.d.
Ac28	"	10	320	640	10	891	1258
Ac26	Vangard®7	40	320	1280	10	446	1258

n.d.= not done

5.3.4 Comparison of sero-response to modified live vaccines between the two otter species

Asian small-clawed otter showed higher VN mean titres after the first (GMT: 794) and second (GMT: 1840) vaccination with MLV vaccine than Eurasian otters (GMT: 66 and 206, respectively) (Figure 20 and Table 23). A general linear model considered the influence of species, age, sex and body mass on the sero-response to MLV vaccine. Neither species ($F_{1,8}$ =3.233, P=0.110) nor sex ($F_{1,8}$=0.396, P=0.547), age ($F_{1,8}$=1.589, P=0.243) or body mass ($F_{1,8}$=1,240, P=0.298) affected the immune response following vaccination.

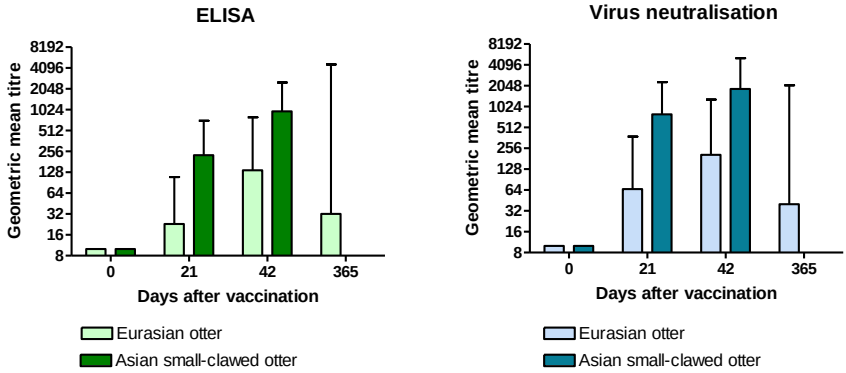


Figure 20 Geometric mean titres induced in Eurasian otters (n=9) and Asian small-clawed otters (n=6) before vaccination (day 0), after the 1st (day 21) and the 2nd (day 42) vaccination and one year after the first vaccination (day 365) with MLV CDV vaccine. Error bars depict 95% confidence interval.

Table 23 Immune response to vaccination with MLV CDV vaccine measured by ELISA and VNT is shown as VN geometric mean titres (GMT) with 95% confidence interval (CI) and % of animals with a titre equal to or greater than 20 (n% ≥20) and with a titre equal to or greater than 80 (n% ≥80) after the first and second vaccination.

Species		Response after 1st vaccination				Response after 2nd vaccination				
	n	GMT ELISA (95%CI)	GMT VNT (95%CI)	n% ≥20	n% ≥80	n	GMT ELISA (95%CI)	GMT VNT (95%CI)	n% ≥20	n% ≥80
Eurasian otter	9	23 (6-58)	65 (14-312)	56	44	8	137 (29-660)	206 (39-1086)	89	68
Asian small-clawed otter	6	226 (106-485)	794 (416-1541)	100	100	5	970 (606-1554)	1840 (1035-3272)	100	100

5.3.5 Influence of vaccine strain on sero-response

5.3.5.1 Chicken-cell culture adapted vaccine strain in Eurasian otters and Asian small-clawed otters

A one-way repeated measures analysis was conducted to compare the sero-response to MLV CDV vaccines containing chicken-cell culture adapted vaccine strains between Eurasian otters and Asian small-clawed otters. Both otter species showed sero-conversion with significantly increasing VN GMTs ($F_{2,12}$=85.146, P=0.001), which did not differ in magnitude between the two species ($F_{(1,6)}$=629.320, P=0.132).

Also, no difference could be detected in the percentage of sero-conversion or percentage of protective titres after vaccination. All Asian small-clawed otters (5/5) and all Eurasian otters (4/4) did seroconvert after two vaccinations with MLV CDV vaccine as measured by VNT and ELISA (Figure 21 and Table 24).

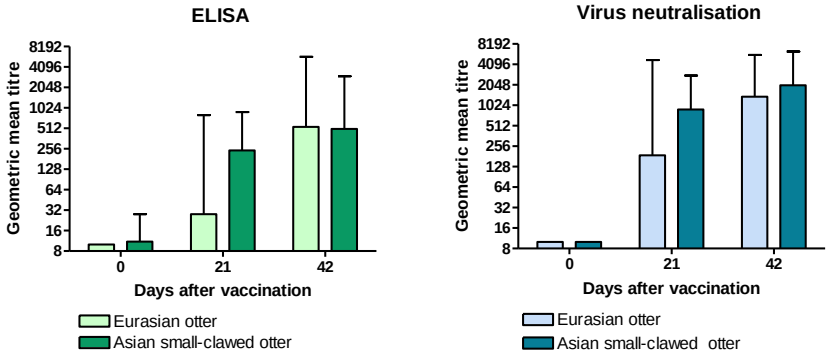


Figure 21: Geometric mean titres induced in Eurasian otters (n=4) and Asian small-clawed otters (n=5) after vaccination with chicken-cell derived vaccine strains (Nobivac® SHP+LT and Virbagen canis® $SH_{(A2)}P/LT^{®)}$. Blood was taken before vaccination (day 0), after the 1st (day 21) and the 2nd (day 42) vaccination. No difference could be detected between the two species. Error bars depict 95% confidence interval.

Table 24 Immune response to vaccination with chicken-cell derived MLV CDV vaccines (Nobivac® SHP+LT and Virbagen canis® $SH_{(A2)}P/LT$) measured by ELISA and VNT is shown as VN geometric mean titres (GMT) with 95% confidence interval (CI) and percentage of animals with a titre equal to or greater than 20 (n% ≥20) and percentage of titres equal to or greater than 80 (n% ≥80) after the first and second vaccination. *P*= probability.

Species	Response after 1st vaccination					Response after 2nd vaccination				
	n	GMT ELISA (95%CI)	GMT VNT (95%CI)	n% ≥20	n% ≥80	n	GMT ELISA (95%CI)	GMT VNT (95%CI)	n% ≥20	n% ≥80
Eurasian otter	4	33 (1-795)	188 (8-4567)	75	50	4	538 (55-5227)	1372 (442-4259)	100	100
Asian small-clawed otter	5	243 (91-647)	890 (417-1901)	100	100	4	501 (101-2486)	2024 (949-4314)	100	100
P				0.444	0.444					

5.3.5.2 Different vaccine strains in Eurasian otters

A one-way repeated measures analysis was conducted to compare the sero-response to vaccination in Eurasian otters between chicken-cell culture adapted vaccine-strain (Nobivac® SHP+LT and Virbagen canis® $SH_{(A2)}P/LT$, n=4) and canine kidney-cell culture adapted vaccine strain (Vangard® 7, n=5). Both vaccine types induced significantly increasing VN GMTs ($F_{2,14}$=21.204, P=0.001) after the first (P=0.016) and second (P=0.016) vaccination. The magnitude in the response differed between the two vaccine strains ($F_{1,7}$=6.965, P=0.033).

The two vaccines containing chicken-cell derived vaccine strains (Nobivac® SHP+LT and Virbagen canis® $SH_{(A2)}P/LT$) induced good immune response after two vaccinations with VN antibody titres ranging from 631 to 2511 (Table 21 and 24). Immune response to the vaccine containing a canine-kidney cell culture derived vaccine strain (Vangard® 7) in contrast was weaker. Of five Eurasian otters vaccinated with Vangard ® 7, two (40 %) did not seroconvert until day 42 post vaccination, and only two otters (40 %) raised high VN antibody titres after two doses of the vaccine (Table 21, Table 22, Figure 22).

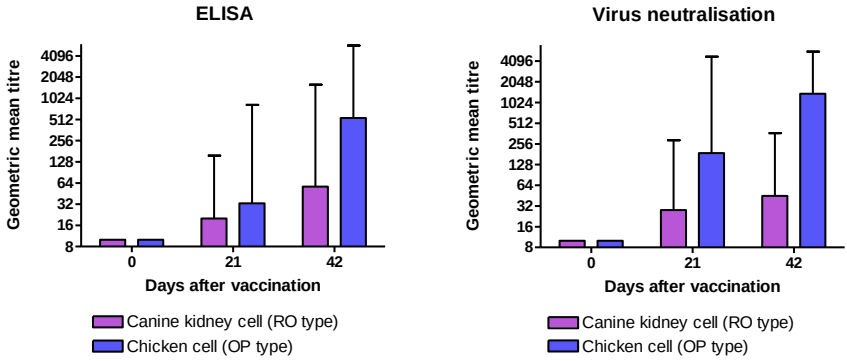


Figure 22 Geometric mean titres in Eurasian otters measured by ELISA and VNT after vaccination with vaccines containing different vaccine strains. Blood was taken prior vaccination (day 0), 3 weeks after the 1st vaccination (day 21) and 3-4 weeks after the 2nd vaccination (day 42). Vaccine containing canine kidney cell derived vaccine strain = Vangard®7 (n=5), vaccines containing chicken-cell derived vaccine strains are Nobivac® SHP/LT (n=3) and Virbagen canis® $SH_{(A2)}$P/LT (n=1). RO= Rockborn type, OP= Onderstepoort type. Error bars depict 95% confidence interval.

Percentage of seroconversion did not differ between the two different strains, not after the first vaccination (Fisher`s exact test, n=8, *P*= 0.524) nor after the second vaccination (Fisher`s exact test, n=8, *P*=0.444). However, percentage of protective titres after two vaccinations was significantly higher when administering chicken-cell derived vaccine strains (Fisher`s exact test, *n*=8, *P*= 0.048) (Table 25).

Table 25 Immune response in Eurasian otters after vaccination with MLV CDV vaccines containing different CDV strains measured by ELISA and VNT is shown as VN geometric mean titre (GMT) with 95% confidence interval (CI) and % of animals with a titre equal to or greater than 20 (n% ≥20) and with a titre equal to or greater than 80 (n% ≥80) after the first and second vaccination. *P* = probability.

CDV strain	Response after 1st vaccination					Response after 2nd vaccination				
	n	GMT ELISA (95% CI)	GMT VNT (95% CI)	n% ≥20	n% ≥80	n	GMT ELISA (95% CI)	GMT VNT (95% CI)	n% ≥20	n% ≥80
Canine Cell (RO type)	5	20 (3-137)	28	40	20	5	57 (2-1547)	45 (6-322)	80	20
Chicken-cell (OP type)	4	28 (1-773	188 (8-4567)	75	75	4	538 (55-5227)	1372 (442-4259)	100	100
P			0.033	0.524	0.206			0.033	0.444	0.048

5.4 Comparison of vaccine types in Eurasian otters and Asian small-clawed otters

Compared to CDV-ISCOM vaccine and MLV vaccine, the immune response to inactivated CDV vaccine was weak. Two doses of inactivated CDV vaccine did not induce sero-conversion in 100% of the Eurasian otters and North American river otters, respectively. In Asian small-clawed otters only 89% of the individuals seroconverted to a low VN GMT (11) after two vaccinations. No VN antibody titres were detectable after one year in all otter species which have been vaccinated with inactivated CDV vaccine (Table 10, Table 11, Table 12).

To compare the immune response in Eurasian otters and Asian small-clawed otters to the three different vaccine types, the VN GMT after two vaccinations was compared using a two-way ANOVA with one factor being the vaccine type (inactivated, ISCOM or MLV vaccine) and the other factor being the species. Virus neutralising GMTs after two vaccinations were significantly different between the three different vaccines ($F_{2,40}$=41.607, P=0.001). Differences were detected between the VN GMT of inactivated CDV vaccine and MLV CDV vaccine (P=0.001) and between MLV CDV vaccine and CDV-ISCOM vaccine (P=0.001). No difference was noticed between the VN GMT of inactivated CDV vaccine and CDV-ISCOM vaccine (P=0.150). Regarding the efficacy of the vaccines, there was no difference observed between the two otter species ($F_{1,40}$=1.159, P= 0.288), but titre values after administration of the different vaccines was species-dependent ($F_{2,40}$=3.981, P=0.027) (Figure 23).

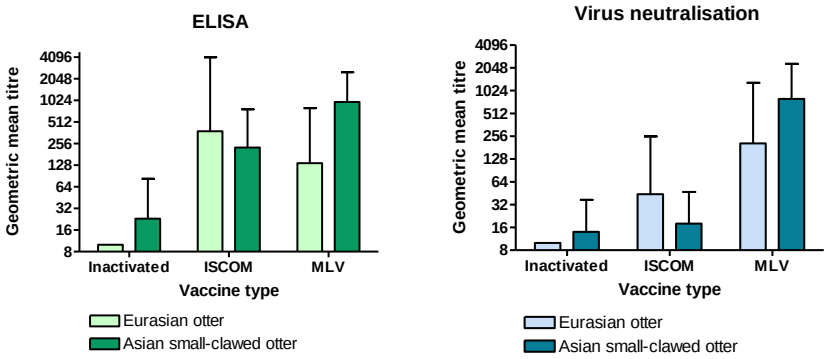


Figure 23 Sero-response in Eurasian otters and Asian small-clawed otters after two vaccinations with different CDV vaccines. Blood was collected 3-4 weeks after the second vaccination. After vaccination with inactivated CDV vaccine hardly a sero-conversion was observed. Vaccination with CDV ISCOM vaccine induced high titres measured by ELISA, but only low to medium VN titres especially in Asian small-clawed otters. High virus neutralising titres were raised after two doses of MLV vaccine in both otter species. Undetectable titres were set as 10.

5.5 Comparison of ELISA and VN

To asses whether it is possible to predict VN titres with an easy and rapidly to perform ELISA, a correlation test and linear regression was conducted. VN titres (y) and ELISA titres (x) after the second vaccination of all Eurasian otters and Asian small-clawed otters of study 1 were used (n=57).

The test revealed a linear relationship. ELISA and VNT antibody titres positively and significantly increased together (Spearmann`s rho=0.754, *P*=0.001). Regression analysis returned moderate r^2 between 0.469 (Asian small-clawed otter) and 0.535 (Eurasian otter), which means that only 46.9% and 53.5%, respectively, of the variance in the VNT titre was explained by the ELISA titre (Figure 24).

The linear regression yielded the following equations for VN titres after the 2nd vaccination

1. VN titre = 0.789 x ELISA titre + 45.399, r^2 = 0.535 (Eurasian otter)
2. VN titre = 1.358 x ELISA titre - 71.789, r^2 = 0.469 (Asian small-clawed otter)

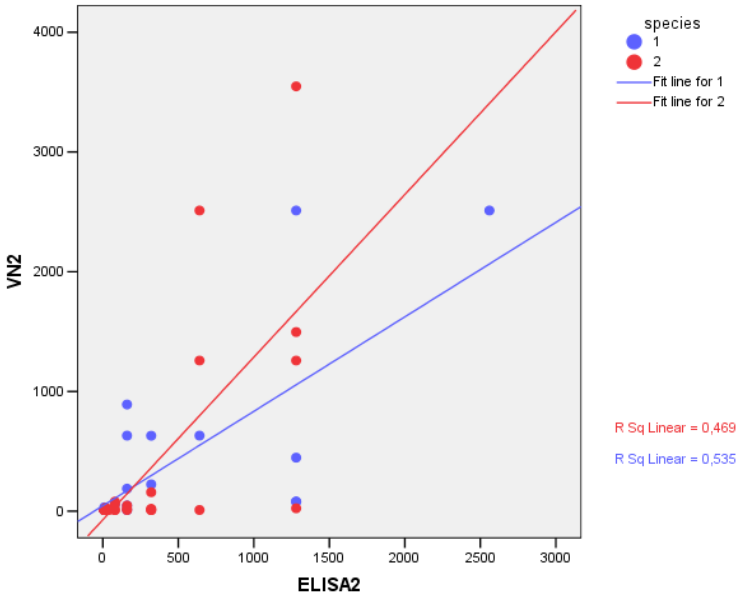


Figure 24 The graph depicts antibody titres measured by VNT after two vaccinations (VN2) versus antibody titres measured by ELISA after two vaccinations (ELISA2). The circles represent values of antibody titres in the different species (blue=Eurasian otter, red=Asian small-clawed otter). The blue and red lines represent the regression line.

Study 2: Booster vaccination

5.6 Booster vaccination with modified live virus vaccine in Eurasian otters

5.6.1 Sample distribution

Eleven adult Eurasian otters, 8 males and 3 females, regularly vaccinated against CDV with different MLV vaccines (Appedix III) were investigated for their CDV-specific antibody titres before and after vaccination with MLV vaccine (Nobivac® SHP+LT). All otters were kept at the Otterzentrum in Hankensbüttel, Germany.

5.6.2 Prevalence of CDV-specific antibodies

All otters had detectable virus neutralising CDV-specific antibody titres before vaccination. Titres ranged from 24 to 224 (GMT VN: 92, 95% CI: 51-164; GMT ELISA: 66, 95% CI: 27-165). High virus neutralising CDV-specific antibody titres (112-224) were observed in 55% (6/11) of the otters. Twenty-seven percent (3/11) had medium virus neutralising titres (40-67),

and low virus neutralising tires (24-28) were detected in 18% (2/11) of the individuals (Table 26).

5.6.3 Sero-response after booster vaccination with modified live virus vaccine

Booster vaccination in Eurasian otters induced a significant rise in VN antibody titres (Wilcoxon signed rank test, *P*(exact)=0.0039, *n*=9). In two (18%) of the otters, which had high VN titres (224) prior to the vaccination, titre values stayed the same. High VN antibody titres (160-630) were observed in 64% (7/11) of the otters, medium VN titres (47-56) were detected in 27% (3/11) and low virus neutralising tires (24-28) in 9% (1/11) of the animals (GMT VN: 121, 95% CI: 62-237). Titres measured by ELISA appeared to be higher than VN titres, with a GMT of 181 (95% CI: 77-426), and ranged between 20 and 1280. One year after booster vaccination, VN titres were still high in 57% (4/7) of the otters (Table 26, Figure 25).

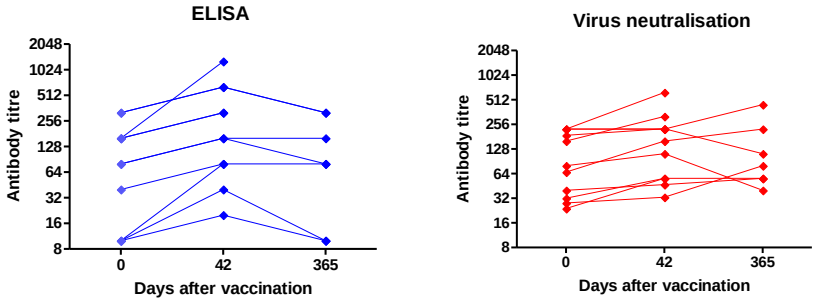


Figure 25 Sero-response in Eurasian otters after booster vaccination with MLV vaccine (Nobivac® SHP+LTLT). Single-case depiction. Dots indicate titres measured by ELISA and VNT before vaccination at times of vaccination (day 0), 6 weeks after the booster vaccination (day 42) and one year after booster vaccination (day 365).

Table 26 Titre values measured in Eurasian otters (n=11) before and after booster vaccination with MLV vaccine (Nobivac® SHP+LT). Blood was collected prior vaccination (day 0), 6 weeks after vaccination (day 42) and one year after the last vaccination (day 365). Titre values <20 were not measurable and are depicted as 10.

Animal ID	Antibody titre ELISA			Antibody titre VNT		
	day 0	day 42	day 365	day 0	day 42	day 365
LL17	40	80	80	28	33	80
LL18	80	160	160	67	160	224
LL19	80	160	80	40	47	56
LL20	320	640	320	224	224	447
LL22	10	20	10	80	112	40
LL23	10	40	10	24	56	56
LL24	0	80	n.d.	32	56	n.d.
LL25	160	320	n.d.	224	224	n.d.
LL26	320	640	320	188	266	112
LL27	160	320	n.d.	160	320	n.d.
LL28	160	1280	n.d.	224	630	n.d.

n.d. = not done

6. DISCUSSION

6.1 CDV vaccination in otters

The emergence of disease in wildlife species caused by morbillivirus infections (Appel et al. 1994) has heightened the need for safe and efficacious CDV vaccines in non-domestic carnivores. Several kinds of CDV vaccines do exist, but hardly any data on its efficacy in non-domestic species is available. Only MLV vaccines, registered for the use in domestic dogs, domestic ferrets or fur animals, are commercially available. However, based on data about vaccine-induced CDV infections in different wildlife species after vaccination with MLV vaccine (Montali 1994), these vaccines cannot be recommended for extra-label use in non-domestic carnivores.

Proper fencing as well as strict hygiene and quarantine protocols could reduce contact to un-vaccinated wild or domestic species and the risk of CD outbreaks in zoo-settings, which occur (Franke et al. 1989; Jens Riege, personal communication) but are relatively rare (Montali et al. 1994). However, all these measures seem to be unable to completely avoid CDV infection. Significant outbreaks of „naturally" occurring CD of unknown source were reported in highly endangered red pandas in Kyoto Municipal Zoo in Japan (Kotani et al. 1989) and in red pandas from Leipzig Zoo in Germany (Eulenberger et al. 1993). The latter was associated with free-living martens (*Martes foina*). CDV outbreaks among captive Eurasian otters and Asian small-clawed otters have been reported from Bronx Zoo, New York (Scott 1979), from a Belgian zoo park (de Bosschere et al. 2005) and from the Tierpark Kunsterspring in Neuruppin, Germany (Jens Riege, personal communication 2009). In the Belgian zoo, acute death over a period of 48 hours was observed in five littermates of Asian small-clawed otters. Neither the puppies nor the parents had been vaccinated against any infectious disease. The animals lived in an isolated pool without contact to other animals (domestic, non-domestic or wildlife CDV-susceptible species) which demonstrates that absolute isolation is difficult to attain. It is probably appropriate to assume a continuous threat of CDV to susceptible captive wildlife species. Outbreaks of CD among endangered species might result in catastrophic losses if these animals are not immunised against CDV (Montali 1994).

The semi-aquatic otters are members of the highly CDV-susceptible mustelid family. They have evolved in habitats that are now frequently threatened (Kruuk 2006). They are specialised foragers, feeding on difficult prey types; much learning is needed to acquire prey, and foraging is energetically so expensive that large quantities of prey have to be caught within a short time. Otters have a fairly risky lifestyle. They are frequently exposed to anthropogenic changes in the environment such as climate change, pollution and over-fishing; with the latter some of their important prey species vanish. The animals are sought after for their fur, they compete for fish with people and some are killed by other predators. And, otter populations have a high mortality rate, increasing with age, and low reproductive rates (Kruuk and Conroy 1991; Kruuk 2006). A small increase in the occurrence of a disease or in a pollutant, a slight decrease in prey populations or an increase in predation might therefore have effects much more dramatic than for other similar-sized species (Kruuk 2006).

Hence, the specialised and vulnerable otters would benefit from a safe, efficacious and long-lasting CDV vaccine, especially if they are part of re-introduction programs upon release in CDV-endemic areas (Melissen 2000). However, CDV vaccination has been problematic in wildlife species and currently there is no safe and effective CDV vaccine commercially available in the EU for use in otters or other highly susceptible non-domestic carnivores. It therefore seemed useful to evaluate the safety and efficacy of CDV vaccines available in Europe (inactivated CDV vaccine, experimental CDV-ISCOM vaccine and MLV vaccine) in otters. Eurasian otters, Asian small-clawed otters and to some extent the North American river otters are the most common otter species found in European zoos and were therefore chosen for this study.

6.2 Statistics

The basic idea of statistics is to make inferences from a sample to the larger population from which the sample was derived. The statistical methods used are based on three assumptions: (1) that an infinite large population of values exists, (2) that the sample was randomly selected, and (3) that each subject was sampled independently of the rest. If these assumptions are fulfilled, the data can be analysed and the rules of probability can be used to make inferences about the overall population (Motulsky 2003).

The best way to use data from a sample to make inferences about the population is to compute a 95% confidence interval (CI). This statistical calculation combines sample size and variability (standard deviation) to generate a range of values for the population mean. In case certain assumptions are accepted (different for the various tests) there is a 95% chance that the 95% CI contains the true population mean (Motulsky 2003).

The population of captive otters is large and blood-samples after vaccination could be taken many times. Theoretically all captive healthy Eurasian otters, North American river otters and Asian small-clawed in Germany and the Netherlands not previously vaccinated against CDV had the same possibility to become part of the study (study 1). However, a selection was done by the directors of the different zoos who accepted taking part in the study or not. As the assumption can be made that the immune response of the otters is random with respect to the acceptance of zoo directors to participate in the study, random selection can be assumed and it seems reasonable to extrapolate from this data to the base population.

The samples needed to be independent from each other. The sampled otters were independent from each other but from each otter data were collected repeatedly. To take this into account, repeated measures analysis was conducted to compare the means of the antibody titres. The repeated measures analysis can be very powerful as it controls for factors that cause variability between subjects. Repeated measures one-way or two-way ANOVA compare three or more matched groups, based on the assumption that the residuals of the model for the differences between matched values are normally distributed. In case of large sample sizes the ANOVA is fairly robust to violation of this prerequisite (Motulsky 2003). Moreover, the Central Limit Theorem of statistics says that if the sample is large enough, the distribution of means will follow a Gaussian distribution even if the population is not Gaussian. Assuming the population does not have a really unusual distribution, a sample

size of 10 or so is generally enough to invoke the Central Limit Theorem (Motulsky 2003). A further assumption is sphericity. This means, that a random factor that causes a measurement in one subject to be a bit high (or low) should have no affect on the next measurement in the same subject. The assumption of sphericity was always met, the assumption of a Gaussian distribution was met in most cases. A small *P* value (≤0.05) will therefore indicate with 95% probability that the confidence interval contains the true difference between two means.

Correlation analysis and linear regression analysis were conducted to examine the relationship between VNT titres and ELISA titres after the second vaccination. The correlation coefficient tests the significance of the relation between two variables. Linear regression finds the line of best fit to predict y from x (Motulsky 2003). The assumption of independent subjects and independently measured ELISA and VNT values for the nonparametric Spaermann correlation coefficient was given, so the conclusions made from the correlation coefficient are valid. The basic requirement for the execution of a linear regression analysis, a linear relationship between the variables, did exist. However, other assumptions such as approximately normally distributed residuals and equality of variances (same standard deviations of the scatter of points along the line) were not met. In such a case, the line of best fit will still be the best predictor of y from x but possible significance tests will be invalid. As in this study the regression analysis only served to make quantitative predictions, the regression results can be used. Given the moderate correlation coefficients and associated r^2 values of the regression equations, predicting VNT titres from ELISA results will be of limited use.

A general linear model was conducted to analyse the influence of sex, species, age and body mass on the immune response of the otters after CDV-ISCOM vaccination. Assumptions for this statistical test, normally distributed data and equality of variances, were met. However, the sample size was small (n=14). The results have therefore to be interpreted cautiously, as the sample size for a GML should usually be five times greater than the number of categories (n=4) used in the model (Bettina Wachter, personal communication).

6.3 Laboratory diagnosis

In non-domestic animals, careful vaccination studies are limited in number and their evaluation is largely restricted to evaluation of humoral responses extrapolated to known challenge infection data from domestic animals (Montali et al. 1983; Hoover 1985, 1989; Williams et al.1996; van Heerderen 2002). For serological diagnostics, the same tests are used in wildlife species as for domestic animals. However, analytical tests are not standardised or validated for the different non-domestic species, thereby hindering evaluation and comparison of vaccine-induced immunity in many different non-domestic species (Philippa 2007). Species-specific differences could lead to different reactions in serological tests (Gardener et al. 1996) and sensitivity and specificity of the single analytical tests are known for only few species (Munson and Cook 1993).

6.3.1 Neutralising peroxidase-linked antibody test

The capability of an animal to produce neutralising antibodies against CDV is directly related to protection in vivo (Murphy et al. 1999) and with survival after infection (Appel 1969; Appel et al. 1982). The VNT is therefore regarded as the “gold standard” for determination of immunity to morbilliviruses (Rikula et al. 2001) because it measures functional neutralising antibodies directed at the haemagglutinin (H) and fusion (F) proteins of the virus (Griffin 2001).

The conventional micro-titre neutralisation test (MNT) is based on CPE readings. It can take up to 7 days until CPEs can be accurately determined. The NPLA assay used in this study has appeared to be at least as sensitive as or even superior to the MNT and delivers results more rapidly (Zaghawa et al.1990).

Antigenetic similarity of IgG between river otters, domestic dogs and domestic cats was shown by Hoover (1985). As all otters are closely related and genetic diversity is low (Kruuk 2006), the similarity of epitopes with those of domestic dogs and domestic cats was assumed also for the otter species included in this study and permitted the utilisation of commercial horseradish peroxidase conjugate against dog IgG for the evaluation of otter sera.

Maximum levels of CDV-specific VN antibody titres are reached 14 to 21 days after vaccination. Therefore, blood was collected at this time and booster vaccination applied. In some serum samples, the Vero cell mono-layers exhibited non-specific cell death (serum toxicity) at low dilutions (<20), which disappeared at higher dilutions. This phenomenon is observed regularly in VNT and might be due to anticoagulants (Haas 2001). Hence, a level of 1/20 was chosen for the first dilution and the production of detectable levels (≥1/20) of VN antibodies after vaccination was taken to indicate the presence of an active immune response with involvement of immune memory.

6.3.2 Indirect ELISA

ELISAs are the serological assays of choice for the qualitative and quantitative determination of viral antibodies (Murphy et al. 1999). The assay is easy and rapidly performed and future evaluation of vaccine-induced antibody titres would be more practical if the ELISA test can be used. Therefore, serum antibody titres were determined by both methods and compared.

Commercially available horseradish-peroxidase conjugated protein A was used to detect CDV-specific immunoglobulin bound to the antigen coated wells. This conjugate is suitable as a second antibody in a great range of wildlife species, including mustelids, and is a useful alternative to species-specific secondary antibodies in various diagnostic assays (Stöbel et al. 2002).

6.4 Sero-response to vaccination

6.4.1 Inactivated CDV vaccine

Inactivated CDV vaccine induced poor immunity based on CDV-specific serum antibodies. No seroconversion was observed after two doses of inactivated CDV vaccine in Eurasian otters and North American river otters sero-negative before vaccination and only two of nine Asian small-clawed otters did seroconvert to low (20) and high (80) VN antibody titres, respectively, following two doses of the vaccine. Antibody titres might have climbed after the third vaccination but would have been of only relatively short duration, as CDV-specific antibodies were absent one year after the initial vaccinations. However, all Eurasian otters sero-positive before vaccination did show good sero-response with high VN antibody titres three weeks after the second vaccination and with detectable VN titres after one year. In sero-positive Asian small-clawed otters, only one of three animals showed a similar reaction to vaccination as sero-positive Eurasian otters, whereas the other two failed to develop an appropriate humoral immune response.

Inactivated vaccines are preferred in cases of safety concerns of the MLV vaccine, as they do not contain infectious virus and are therefore incapable of causing an infection. Inactivated vaccines act as exogenous antigens, triggering an immune response dominated by CD4+ and often Th2 cells, which may not always be the most effective response to the pathogen vaccinated against. Additionally, the process of inactivation may dramatically reduce immunogenicity, usually resulting in an immune response that is shorter in duration, narrower in antigenetic spectrum, weaker in cell-mediated and mucosal immune responses, and less effective in preventing viral replication (Murphy et al. 1999).

To maximise the effectiveness of vaccines, especially those containing killed organisms, it has been common practice to add so-called adjuvants to the antigen. Adjuvants can greatly enhance the host`s response to vaccines and are essential if long-term memory is to be established. Their mode of action is only poorly understood but in general they work through one of three mechanisms: (1) Depot adjuvants simply protect antigens from rapid degradation and so prolong immune responses. This is the case for aluminium hydroxide used as adjuvant in the inactivated vaccine administered in this study. (2) Particulate adjuvants are effective at delivering antigens to antigen-presenting cells. (3) ISCOMS which are discussed in chapter 6.4.3 are complex lipid-based micro particles. And immunostimulatory adjuvants consist of molecules that enhance cytokine production. However, the use of adjuvants can also cause severe inflammation and systemic toxicity and repeated or high doses can induce hypersensitivity reactions (Tizard 2006).

Inactivated CDV vaccines do not produce sufficient immunity to prevent infection after challenge exposure in domestic dogs, but show less severe disease in vaccinated domestic dogs than in unvaccinated controls (Greene and Appel 2006). In non-domestic species, inactivated CDV vaccines have shown good safety but variable efficacy (Montali et al. 1998), which might be explained by the different action of mechanism of the different adjuvants used and variable reactions of different wildlife species to vaccination. African wild dogs, for example, vaccinated with an inactivated CDV vaccine containing aluminium-hydroxide as

adjuvant, showed no seroconversion for VN CDV specific antibodies, in contrast to African wild dogs vaccinated with inactivated CDV vaccine with an added oil adjuvant (Cirone 2004).

The formalin-inactivated vaccine used in this study has been used in German zoos since the 1980s in many carnivore species, including Eurasian otters. The vaccine always was well-tolerated and no side-effects were observed. VN antibody titres induced in the different species varied and ranged from 0 to 1:237 (Franke et al. 1989). Hybrid ferrets (black-footed ferret x Sibirian polecat, n=8) vaccinated with inactivated CDV vaccine (beta-propiolactone inactivated) and adjuvant (Stimulin, Fort Dodge Laboratories, Fort Dogs, Iowa) developed VN CDV-specific antibody titres 21 days post vaccination ranging from 1:32 to 1: 1024 (Williams et al. 1996).

Vaccination results achieved in this study with inactivated CDV vaccine in otters are comparable to the results described by other authors (Montali et al. 1983; Franke et al. 1989; Cirone et al. 2004). The results indicate that the formalin-inactivated CDV vaccine available in Germany is not very effective in otters. Vaccination failure caused for example by interruption of the cold chain or incorrect administration can be excluded, as the vaccine and the otters that did not seroconvert were handled exactly in the same way as the otters sero-positive before vaccination which showed good increase in VN antibody titres. The complete lack of CDV-specific serum antibody titres as measured by ELISA and VNT in Eurasian otters, North American river otters and most of the Asian small-clawed otters, might therefore suggest that these species are low responders to inactivated CDV vaccine containing aluminium hydroxide as adjuvant and repeated boost vaccinations would have been necessary to induce an immune reaction. However, as the used vaccine belongs to the last remnants of the German stock of inactivated CDV vaccine with unknown production date, the vaccine might have been beyond its durability date and has maybe lost some function ability, so the dose given was not sufficient to induce sero-response. This assumption is supported by the reaction of otters sero-positive prior to vaccination which showed a good immune response.

The presence of CDV-specific antibodies in two 3-month old Asian small-clawed otters (Ac6, Ac7) and their low or lack of response to vaccination, respectively, might be due to persisting maternal antibodies. Pre-vaccination CDV-specific serum-antibody titres in the other otters, all adult, might be attributed to unrecorded prior vaccination, or alternatively to previous exposure to virulent CDV by contact to CDV-susceptible wildlife species. As all otters live in outdoor enclosures both scenarios are possible.

6.4.2 CDV ISCOM vaccine

Two doses of CDV-ISCOM vaccine given at a three-week interval induced medium VN CDV-specific antibody titres (GMT: 44) in Eurasian otters and low VN serum antibodies (GMT: 16) in Asian small-clawed otters. Percentage of seroconversion in Eurasian otters and Asian small-clawed otters until day 42 post-vaccination was 75% (3/4) and 25% (2/8), respectively. One year post-vaccination, VN GMTs had increased in Asian small-clawed otters, suggesting that the antibody responses were boosted by the administration of the third vaccine dose and probably adequate VN titres would have been reached after the third dose, although no

blood was collected at the time of the expected peak in antibody titres. But nevertheless, one of the Eurasian otters and two of the Asian small-clawed otters failed in developing VN antibody titres at all, indicating a poor immune response to CD ISCOM vaccine in these species.

Surprisingly, vaccination did induce much higher antibody titres detected by ELISA (GMT: 381 and GMT: 226 for Eurasian otters and Asian small-clawed otters, respectively, at day 42 post vaccination) with 100% of the otters showing titres > 80 after two vaccinations. The discrepancy between VN and ELISA titres was especially pronounced in Asian small-clawed otters. One year post-vaccination 50% of the Eurasian otters and 67% (6/9) of the Asian small-clawed otters had still titres >80 as measured by ELISA.

These results are similar to results found in African wild dogs vaccinated with CDV-ISCOM vaccine (Philippa 2007) but in strong contrast to a similar vaccination evaluation in Eurasian otters (n=2) and Asian small-clawed otters (n=3) with CDV-ISCOM vaccine conducted at Rotterdam zoo (Philippa 2007). In this study both otters species showed high antibody titres measured by VNT and ELISA, whereas in the African wild dogs only low antibody titres were induced and a big discrepancy between ELISA and VN titres was noticed. This suggests that the poor sero-response for VN antibodies in this study in otters is not necessarily due to otters being low responders to CDV-ISCOM vaccine, as it was assumed in African wild dogs. A different batch of the vaccine was used in Rotterdam, but the dose of antigen was the same (10µg/ml), as was the ELISA used and the way the VNT was conducted. A quality-reducing interruption of the cold chain required for preservation of the CDV-ISCOM vaccine can be excluded. Age of the otters might be an issue. Four of the Asian small-clawed otters were 12 weeks at the time of the first vaccination. But, in all of these otters, high ELISA antibody titres were induced and in three of the otters, VN antibody titres ranging from 40 to 112 were observed one year after vaccination. Alternatively, a loss of immunogenicity of the CDV antigen during ISCOM preparation may have rendered it sub-optimal in terms of inducing a humoral response in the Eurasian and Asian small-clawed otters, or results might have simply been caused by individual variation in immune response.

Antibody titres measured by ELISA can be expected to be higher than titres measured by VNT, because the ELISA measures antibodies directed at a much larger range of epitopes than the VNT test (Philippa 2007). The VNT measures functional neutralising antibodies directed at the H and F surface proteins of the virus (Philippa 2007). The ELISA, which is based on Vero cell culture-grown detergent-treated virus antigens, detects antibodies directed at the H and F surface proteins of the virus and it also detects antibodies against the nucleoprotein (NP) antigen and possibly cell components. Antibodies to NP do not contribute directly to neutralisation and are therefore missed in a VNT. Nevertheless, antibodies against these structures are abundantly produced in response to infection or vaccination with conventional vaccines (Cohen et al. 2006), although not in response to the ISCOM vaccine (Philippa 2007). The ISCOM vaccine uses virus grown on Vero cells as used in the ELISA. It is produced in a manner that incorporates the H and F proteins into the ISCOMS and then purified, leaving little or no NP or cell components in the vaccine. The presence of small amounts of NP or cell components in the vaccine may induce additional antibodies against these epitopes, which can be measured by ELISA. However, the inclusion of an ELISA

coated with an uninfected Vero cell lysate used as a control for these sera excludes serum antibodies directed against the Vero cell components.

Using vaccine-induced VN antibodies as correlates of protection, CDV-ISCOM vaccine seems to be not very efficacious in Eurasian and Asian small-clawed otters. However, the rise in antibody titres measured by ELISA shows clearly that an immune response has occurred. Effective vaccination induces not only a humoral but also a cellular immune response (Tizard 2004). T cell responses are likely to be of importance in providing immunity to infection with CDV, and it has been suggested that these vaccine-induced cell-mediated responses complement humoral immunity and result in adequate protection in the absence of high serum antibody titres (Stephensen et al. 1997; Pardo et al. 1997). ISCOMs have been shown to induce specific T-cell responses in macaques (Rimmelzwaan et al.1997) and CDV-ISCOM vaccination has been shown to be safe and protective in harbour seals after challenge with the closely related PDV (Osterhaus et al. 1989). Therefore, it cannot be excluded that the CDV-ISCOM vaccine used in this study would have induced protective immunity in these species.

6.4.3 Modified live virus vaccine

As MLV vaccines, especially the canine-cell-adapted vaccines, have caused fatal CDV infections in several wildlife species (Greene and Appel 2006), the safety of these vaccines was an important concern. Modified live virus vaccines have been so far used in captive Eurasian otters, North American river otters and Asian small-clawed otters without any problems (Oaf Behlert, Peter Drüwa, Hans-Henry Krüger, personal communication; Hoover 1986; Bosschere et al. 2005), so the risk in using this type of vaccine in otters was considered to be low. Two of the vaccines used were of egg-adapted type, one of canine-cell adapted type and no adverse or side effects were observed in either species with any of these vaccine types.

Eleven Eurasian otters were vaccinated with MLV CDV vaccine. Two of the otters showed high VN antibody titres prior to vaccination. One of the initially sero-positve otters (8 weeks old) had no detectable antibodies at day 21 and day 42 after vaccination, suggesting that antibodies detected initially were passively acquired from the dam rather than a product of active immunity. The presence of antibodies in the other otter may be attributed to unknown vaccination before or alternatively to previous exposure to virulent CDV virus by contact to CDV susceptible wildlife species. As this otter was found as a juvenile in the wild and as it is housed in an outdoor enclosure the latter scenario might be more likely.

Vaccination with MLV CDV vaccine revealed a difference in sero-response depending on the vaccine strain used. Eurasian otters vaccinated with the canine-kidney cell adapted vaccine strain (Vangard®7) developed significantly lower VN GMTs (GMT: 45) than otters vaccinated with chicken-cell adapted CDV vaccine strains (Nobivac®SHP+LT and Virbagen canis®$SH_{(A2)}$P/LT) (GMT: 1372). Both otter species developed high VN antibody titres after vaccination with chicken-cell adapted strains, whereas the canine-kidney cell adapted strain induced only low or medium VN GMTs in Eurasian otters, with one of the otters not seroconverting at all.

Different vaccine strains produce different levels of protection in dogs (Rikula 2000) and similar results were seen in vaccination studies conducted in North American river otters and domestic ferrets. North American river otters (n=20) vaccinated with two doses of commercially available MLV CDV vaccine in a two week interval did not seroconvert until day 21 post vaccination (Hoover et al. 1985). Titres in this study were determined by VN. The vaccine used contained a canine kidney cell attenuated CDV strain (Vangard® DA_2PL), as does the Vangard®7 vaccine. In contrast, three North American river otters which had received a chicken-cell attenuated MLV CDV vaccine (Fromm-D, Solvay) did seroconvert (Petrini 1992). In another study (Hoover et al. 1989) the level of antibody production elicited by chicken-cell attenuated MLV CDV vaccine in hybrid ferrets and domestic ferrets was higher and of longer duration than that reported in domestic ferrets following vaccination with a canine cell origin multivalent MLV vaccine (Vangard DA_2PL). Vaccination of five ferrets with three doses of MLV vaccine containing a CDV strain of chicken cell origin (Biocan Puppy and Biocan DHPL) in a 30 days interval led to high VN antibody titres in only three ferrets whereas two ferrets did not seroconvert (Pavlazik et al.2007).

In domestic dogs, canine-cell adapted CDV strains are more immunogenic than chicken-cell adapted vaccines and induce high titres of VN antibodies and long-term protection (Greene and Appel 2006), in contrast to chicken-cell adapted strains which may produce lower levels of immunity (Appel 1973). So, if the above mentioned otter species and domestic ferrets are low responders to canine-cell-adapted MLV vaccines, then this would contrast with the response in domestic dogs. Immunogenicity of a vaccine depends not only on virus attenuation but also on passage level and method of production (Rikula 2001) and in general, comparison of vaccine-induced immunity between non-domestic species and domestic dogs is hindered by the fact that analytical tests are not standardised or validated for different species. Whether differences between vaccine strains explain the differences observed in immune response of Eurasian otters to MLV CDV vaccine needs further research.

6.5 Comparison of ELISA and VN test

In general, VNT titres and ELISA titres seemed to follow a similar trend but with ELISA titres being consistently higher. As previously stated in the discussion on antibody titres after ISCOM vaccination (Chapter 6.4.2), antibody titres measured by ELISA can be expected to be higher than by VNT because the ELISA measures antibodies directed at a much larger range of epitopes than the VNT test. However, the discrepancy observed between titres, especially after CDV-ISCOM vaccination, was quite high as discussed in chapter 6.4.2. Regression analysis between VNT antibody titres and ELISA titres after two vaccinations yielded only moderate r^2 of 0.469 for Asian small-clawed otters and 0.535 for Eurasian otters, respectively. This suggests that the fit between both methods is not very tight and inspection of figure 24 demonstrates that the fit is probably not tight enough to provide a practical method to extrapolate from one method to the other. The ELISA titre demonstrates that immune response has occurred, so this test might be suitable for screening purposes, but titre values measured might not be useful to predict protection against CDV.

As VNTs are cost and time-consuming, several approaches have been made to develop more convenient ELISA techniques for the detection of CDV-specific antibodies (Blixenkrone-Möller et al. 1991; Gemma et al. 1995) which showed promising results with respect to sensitivity and specificity as compared to those of the VNT. A capture-sandwich ELISA that uses recombinant baculovirus-expresed N protein of a recent CDV wild-type isolate showed a very good inter-rate agreement (kappa=0.988) indicating that this ELISA can be unrestrictedly used as a substitute for the qualitative determination of CDV-specific IgG serostatus (von Messling et al. 1999). Noon et al. (1980) noticed a high degree of correlation with 97% agreement within a fourfold dilution between ELISA and VNT antibody titres. Waner et al. (1998) used a semiquantitative microenzyme-linked immunosorbent assay (dot-ELISA) to asses CDV-specific antibody titres and noticed a good correlation (r^2=0,748). However, findings after CDV-ISCOM evaluations by Philippa (2007) were similar as in this study. The ELISA used in this study was the same as in the vaccination study of Philippa . So the discrepancy in correlation between antibody titres measured by ELISA and VNT in the different studies might be due to the different ELISA techniques used and will need further research.

6.6 Factors influencing sero-response

There are many factors which might influence sero-response after vaccination (see chapter 2.2.4). A general linear model was conducted to evaluate the influence of age, sex, species and body mass to sero-response after vaccination with MLV CDV vaccine. None of these factors seemed to have affected the sero-response of the Eurasian otters and Asian small-clawed otters.

Age will influence the immune system both early and late in life, and can therefore be expected to influence the efficacy of a vaccine (Rikula 2008). The immune system of a young animal may not function as efficiently as that of an adult individual (Tizard 2004) and during ageing, both cellular and humoral immune responses will be impaired (Gerber and Brown 1974; Kennedy et al. 2007). Human neonates are not able to respond to most polysaccharide antigens and the response to protein antigens continues to further maturate during the first years of life (van Loveren et al. 2001). This might be similar in young mammals. Furthermore, maternal antibodies will protect young animals in the first weeks or month against infectious diseases and may interfere with MLV vaccines (Tizard 2004). Maternal antibodies are considered to be the most important cause of vaccine failures. And the influence of these has been described in domestic dogs and non-domestic carnivores in many studies (Paré et al.1999; Böhm et al. 2004; Kennedy et al. 2007). Rikula (2008), however, noticed no maternal antibodies in beagles of 12 weeks of age and no serious influence of maternal antibodies on seroconversion in American mink and silver foxes, respectively, after vaccination, concluding that maternal antibodies are perhaps a less important cause of vaccine failure than claimed, provided the current vaccination recommendations are followed. Furthermore, such passive antibodies wane with age and will be undetectable in domestic ferrets and domestic dogs by 12 weeks of age (Appel and Harris 1988; Stephensen et al. 1997; Rikula 2008) and in raccoon pups by 20 weeks of age (Paré et al. 1999). Findings in this study are in agreement with vaccination studies in domestic ferrets (Stephensen et al. 1997), maned wolves (Maia and Gouveia 2001) and domestic

dogs or fur animals (Eghafona et al. 2007; Rikula 2008) but in contrast to a vaccination study in raccoon pups (Paré et al.1999) vaccinated with MLV CDV vaccine, where 7 out of eight 8-week-old pups showed high maternal antibody titres and failed to seroconvert. The reason for this discrepancy might be due to difference in age or antibody amount the animal had received and maybe due to differences in decline of maternal antibodies, although the half-life of maternal antibodies was reported to be similar for domestic dogs, domestic ferrets and raccoons (Appel and Harris 1988; Paré et al. 1999):

There is no report of sex influencing the response to CDV vaccination in non-domestic carnivores. However, in humans there is evidence of sex differences in the humoral immune response to high titre measles vaccines (Greene et al. 1994) and the immune response to hepatitis B vaccines (Corrao et al. 1988). There seem to be a significantly greater humoral antibody response in females after vaccination than in males. The mechanisms underlying the observed sex difference is not clear. The genetic control of immunoglobulins has been shown to be associated with the X chromosome (Rhodes et al. 1969) and also a possible effect of androgens and estrogens on the immune system cannot be excluded (Green et al. 1994). Whether an effect like this exists in response to CDV vaccination in carnivores remains to be tested.

Species identity will influence immune response to CDV vaccination, as it is already reflected in the variation in susceptibility to the disease in the different carnivore species (Deem 2000) and in the difference in sero-response between species (Franke et al. 1989; Rikula et al 2001; Philippa 2007). Closely related species are assumed to react similarly to CDV vaccination, which is supported by the results seen in this study. However, different breeds of dogs, all very closely related, have been shown to significantly vary in median titres after CDV vaccination (Rikula et al. 2001) or rabies vaccination (Kennedy et al. 2007). This might be due to their genetic profile or other characteristics such as size or age.

A general relationship between body mass and level of antibody response clearly exists in domestic dogs (Mansfield et al. 2004). Smaller sized dogs elicit higher antibody levels than larger breeds of dogs (Kennedy et al. 2007). The reason for this is not yet clear. An assumed vaccine-dose effect seems unlikely since as long as the immune system of an animal encounters sufficient antigen to make a response, larger doses of antigen are not a major factor in increasing antibody production in primary responses (Kennedy 2007). The enormous difference in body mass between small and large breeds of dogs might be an explanation for seeing no influence of body mass to the immune response in this study. Eurasian otters are indeed bigger than Asian small-clawed otters. However, the average difference in body mass between the two otter species was 3-4 kg, which is not much in comparison to the difference of 30-40 kg between small and large breeds of dogs. There are no reports of body mass influencing immune response following vaccination in non-domestic carnivores.

6.7 Booster vaccination

Eurasian otters regularly vaccinated against CDV showed pre-vaccination VN antibody titres ranging from 1:24 to 1:224. Booster vaccination induced a significant rise of VN antibody titres in all otters, with high VN antibody titres in 67% of the animals measured at day 42 post vaccination. All otters evaluated after one year had medium or high VN antibody titres, suggesting protection against CDV. In dogs, VN antibody titres decrease with older age and with the number of years since boosters had been given (McCaw et al. 1998), so it could be assumed that the otters with medium VN antibody titres were protected against CDV. Similar results were seen in other carnivores following vaccination with MLV CDV vaccine of Onderstepoort type, such as American badgers (Goodrich et al. 1996) and gray foxes (Halbrooks et al. 1981). Titres, however, raised in domestic ferrets and hybrid ferrets (black-footed ferret x Sibirian polecat) (Williams et al. 1996) were higher and high levels were sustained until the end of the study at day 791. Twark and Dodds (2000) evaluated 1379 domestic dogs for antibody titres. Nearly all dogs (96%) had adequate VN CDV-specific antibody titres (≥100). The percentage measured in Eurasian otters in this study was lower in comparison under the assumption of an adequate titre of ≥80. If it is assumed that medium titres are protective, which is conceivable, results seen in this study are similar.

6.8 Comparison of vaccine efficacy in the two otter species

There was no difference between the two otter species regarding the efficacy of the three vaccine types, which was discussed in chapter 6.4.2, but the efficacy of the vaccines was significantly different. Only CDV-ISCOM vaccine and MLV CDV vaccines were able to induce antibodies against CDV. Inactivated CDV vaccine did not induce seroconversion in any of the Eurasian otters or North American river otters and also not in 89% of the Asian small-clawed otter. The proportion of animals with detectable antibody levels in a group is regarded an important measure of vaccine efficacy. In the case of measles, 91-95 % of a group need to have protective titres to provide herd immunity (Nookes and Anderson 1988; Woolhouse and Bundy 1997). If a large proportion of a group of immuno-competent animals has not produced neutralising antibodies within 4-6 weeks after vaccination, it is questionable whether the vaccine is immunogenic enough (Rikula 2001). In this respect, inactivated CDV vaccine did not perform well in the species tested. Virus neutralising GMTs induced by two doses of MLV vaccine were in general higher than those induced by CDV-ISCOM vaccine. Also, the percentage of otters with high VN antibody titres was higher after receiving MLV vaccine. These findings are in disagreement with results obtained after CDV-ISCOM vaccination in Eurasian otters and Asian small-clawed otters in another vaccination study (Philippa 2007), which was discussed in chapter 6.4.2. ISCOM vaccines have shown to be potent inducers of antibody and cell-mediated immune responses and have also proved to induce high levels of protection against infection in many virus systems (for a review see Rimmelzwann and Osterhaus 1995b and Morein et al. 2004). In seals, CDV-ISCOM vaccine induced VN serum antibody titre levels comparable to levels reached after MLV CDV vaccination (range: 1:300-1:1000) (Osterhaus et al. 1989). However, CDV-ISCOM vaccine has also led to catastrophic losses in Afrian wild dogs after vaccination due to insufficient induction of VN antibodies (Vissee 2001; van de Bildt 2002). The African wild dog seems to be a low responder to CDV-ISCOM vaccine, as was shown in a study where African wild

dogs, red pandas, maned wolves, malay civets and European minks were vaccinated with CDV-ISCOM vaccine (Philippa 2007). Only low VN antibody tires were observed after two vaccinations in the African wild dogs, whereas the other species showed high VN antibody titres after two vaccinations which had declined after one year but remained still on adequate levels. Why CDV-ISCOM vaccine induced only moderate VN antibody titres in Eurasian otters and Asian small-clawed otters in this study remains unclear and underlines the need for further research of vaccine efficacy in non-domestic carnivores.

7. Conclusions and perspectives

In the past decades CDV has caused major epidemics in free-ranging terrestrial and marine carnivores as well as in captive highly endangered species such as the black-footed ferret and the African wild dog. Minor outbreaks of CDV have occurred among different captive otter species (de Bosschere 2008, Riege personal communication). The occurence of vaccine induced CD in wildlife species after administration of commercially available MLV vaccines has made CD vaccination problematic, especially in Europe, as there is currently no safe and efficacious CDV vaccine registered for the use in non-domestic species.

In this thesis CDV vaccines available in Europe were evaluated for use in Eurasian otters, Asian small-clawed otters and North American river otters.

All vaccines used were safe. Inactivated CDV vaccine did not induce antibodies against CDV in Eurasian otters and North American river otters. In Asian small-clawed otters, only low antibody titres were raised. The use of formalin-inactivated CDV vaccine in otters seems therefore questionable.

CDV-ISCOM vaccination induced humoral immune response in all Eurasian otters and Asian small-clawed otters, but VN antibody titre levels induced were not indicative of protection. However, as a similar vaccination study (with smaller sample size) has shown adequate levels of VN antibody titres in Asian small-clawed otters and Eurasian otters (Philippa 2007), as high ELISA titres were induced and as VN antibody titres had increased one year after the initial vaccinations, it cannot be ruled out that CDV-ISCOM vaccine might be a safe and efficacious alternative to MLV vaccine in otters.

After vaccination with MLV vaccines, a difference was noticed in sero-response depending on the vaccine strain used. MLV CDV vaccines containing a chicken-cell culture adapted vaccine strain (Onderstepoort type) were safe and efficacious in Eurasian otters and Asian small-clawed otters and induced high VN antibody titres in both species, whereas canine-kidney cell culture adapted MLV CDV vaccine (Rockborn type) was not very effective in Eurasian otters. The use of MLV vaccine of Onderstepoort type is therefore recommended in the tested otter species. One dose of the vaccine seems to be sufficient in Asian small-clawed otters. Eurasian otter might benefit from a second dose after 3-4 weeks. Based on the results in this study and the absence of reports of MLV vaccine-induced CD in otters, the use of MLV CDV vaccines containing chicken-cell culture adapted strains seem to be safe in otters. To minimise risks combined with the use of MLV vaccines not registered for the use in non-domestic carnivores, monovalent vaccines or vaccines with few combinations are recommended.

The correlation between ELISA titres and VNT titres was not very tight. In general, ELISA titres tended to be higher but this was not reliably predictable. The findings suggest that the indirect ELISA tested in this study cannot be used as an alternative for the VNT test with regard to indicating protective titres, although the presence of antibody titres measured by ELISA are indicative of an immune response that has taken place.

Further vaccination studies are recommended to get an increased knowledge of the effect of CDV vaccines in the different CDV susceptible species. Modified live virus vaccines could be evaluated in species with no reports of vaccine induced disease. The role of the vaccine strain needs further research. Safe alternatives like the CDV-ISCOM vaccine and the recombinant canary-pox vectored CDV vaccine could be evaluated in highly endangered species in which vaccine-induced CD has occurred. The reason for the observed discrepancy between ELISA and VNT needs to be studied. Moreover, since in CDV infection the cellular immune response plays an important role (Murphy et al. 1999), studies on cellular immune response after vaccination with CDV vaccine would be very useful, especially in cases where vaccination fails to induce antibody titres.

The disadvantages of administering CDV vaccines in wildlife species by injection (stress of capture and handling, potential for local or systemic reactions, time required, staff cost, staff safety), has led to investigations of oral administration in domestic ferrets and Siberian polecats with promising results (Welter et al. 1999; Wimsatt et al. 2003; Vickers et al. 2004). Vaccine-laced bait has been used to effectively reduce labor costs, capture stress and time required to accomplish vaccination in some wildlife disease management efforts (Selhorts et al. 2001; Knobel et al. 2003; Vos 2003). An oral vaccine would have the advantage to vaccinate also free ranging wildlife species, which could maybe help to reduce drastic CDV epidemics such as the oral rabies vaccine did in continental Europe by large-scale annual campaigns of fox oral vaccination (Wandeler 1991).

In otters MLV CDV vaccine seems to be safe. But nevertheless, currently there is no safe and efficacious CDV vaccine authorised in the EU for use in non-domestic species. CDV-ISCOM vaccine might be an option in other species, but CDV-ISCOM vaccine is an experimental vaccine not registered and only produced in small amounts. The safe and efficacious canary-pox vectored CDV vaccine Purevax® Ferret Distemper (Merial Limited. Duluth, GA, USA) registered for the use in ferrets in the USA and recommended by the AAZV for the use in all CDV- susceptible wildlife species is not authorised in the EU (Philippa 2007). To protect otters and other CDV-susceptible species in the EU against CDV infection, the canary-pox vectored vaccine or another safe and efficacious CDV vaccine should be registered as soon as possible for use in non-domestic species in EU member states.

8. Summary

Humoral immune response in Eurasian otters, Asian small-clawed otters and North American river otters after vaccination with different canine distemper virus vaccines

Canine distemper, a highly contagious systemic disease in dogs and other carnivores is considered to be the most serious infectious disease in mustelids. Susceptible to the causative morbillivirus (CDV) are all carnivores, but mortality and morbidity greatly vary between families and species. Mortalitiy rate in black-footed ferrets and domestic ferrets reaches 100% and vaccination against the disease in captive mustelids is highly recommended. Eurasian otters and Asian small-clawed otters are among the most frequently exhibited mustelids in European zoos and in zoos live in close contact to many CDV susceptible species. Unfortunately, CDV vaccination has been problematic in non-domestic carnivores and few studies on the effect of CDV vaccination have been conducted in otters and other non-domestic carnivore species. Modified live virus (MLV) vaccines developed for the use in domestic dogs or fur animals have induced disease in several non-domestic species, including close relatives of the otters such as the black-footed ferret, the domestic ferret and the European mink. Furthermore, these vaccines have not always been effective in mustelids, including otters. Hence, safe and effective alternatives such as inactivated CDV vaccines, CDV-ISCOM vaccines or recombinant vaccines are recommended. However, currently there is no safe and effective commercially available CDV vaccine for use in non-domestic carnivores in Europe.

In this thesis, the efficacy and safety of CDV vaccines available in Europe were evaluated in Eurasian otters, Asian-small-clawed otters, and to some extent in North American river otters. Two methods of antibody determination were compared. The virus neutralisation test (VNT) is considered to be the gold standard for determining immunity to morbilliviruses (Appel 1973; von Messling et al. 1999), but takes time and is expensive. An easy and rapid to perform ELISA would be very helpful. Seventy otters originating from 19 different zoos in Germany and the Netherlands of both sexes and ranging in age between 8 weeks and 17 years were vaccinated. Two studies were conducted. In study 1, previously unvaccinated Eurasian otters (n=27), Asian small-clawed otters (n=28) and North American river otters (n=4) were vaccinated either with inactivated CDV vaccine, CDV-ISCOM vaccine or with one of three different MLV vaccines. In Study 2, Eurasian otters (n=11) regularly vaccinated against CDV before the onset of this study were evaluated for their CDV-specific VN antibody titre one year after the last vaccination and for their sero-response following booster vaccination.

The results were as follows: None of the otters showed clinical signs of CDV infection. Immune response to inactivated CDV vaccine was low. Two doses of the vaccine did not induce CDV-specific antibodies in Eurasian otters and North American river otters and only low virus neutralising (VN) geometric mean titres in Asian small-clawed otters. CDV-ISCOM vaccine induced humoral immune response in all otters, but high titres were only measured by ELISA. However, considering the increased VN geometric mean titre in Asian small-clawed otters one year after vaccination, a possible cellular immune response and the

induction of protective titres in these species in a similar vaccination study, it cannot be excluded that the CDV-ISCOM vaccine would have induced protective immunity. Vaccination with MLV vaccine revealed that the sero-response depended on the vaccine strain used. Eurasian otters vaccinated with a canine kidney cell adapted vaccine strain (Rockborn type) developed significantly lower VN geometric mean titres than Eurasian otters vaccinated with chicken-cell adapted CDV strains (Onderstepoort type). Both otter species developed high VN antibody titres after vaccination with chicken-cell adapted strains. Age, sex, species and body mass did not influence immune response to MLV vaccine vaccination. The three different vaccine types (inactivated, ISCOM, MLV) showed a significant difference in efficacy with MLV vaccines from Onderstepoort type yielding the best results. There was no difference in geometric mean titres after two vaccinations between the Eurasian otter and Asian small-clawed otter but the development of titres over time was species-dependent. The fit between the two methods, the goldstandard VNT and the quick ELISA was not very tight. Booster vaccination in Eurasian otters with MLV vaccine (Onderstepoort type) induced a significant rise in VN antibody titres, with VN antibody titres staying on an adequate level for up to day 365 post vaccination.

The results led to the following conclusions: The use of formalin-inactivated CDV vaccine containing $Al(OH)_3$ as adjuvant seems at best ineffective and questionable in the tested otter species. Modified live virus CDV vaccines of Onderstepoort type are efficacious in Eurasian otters and Asian small-clawed otters and seem to be safe in these species. The experimental CDV-ISCOM vaccine is safe and might be an efficacious alternative. For both vaccines, a yearly booster vaccination is suggested. The ELISA used in this study is useful for screening purposes but is not recommended as an alternative for the VNT. Further studies are needed to understand the effects of CDV-ISCOM vaccine in otters and other CDV susceptible species and the variation in the effect of different vaccine strains in MLV vaccines. Although, the use of modified live virus vaccine has not been problematic in otters, there is still a risk in using domestic dog vaccines in non-domestic carnivores and the registration of a safe and efficacious CDV vaccine for use in wildlife species in the EU is urgently needed. An oral vaccine would be desirable.

9. Zusammenfassung

Humorale Immunantwort bei Europäischen Fischottern, Asiatischen Kurzkrallenottern und Nordamerikanischen Fischottern nach Impfung mit unterschiedlichen Staupeimpfstoffen

Staupe, eine hochinfektiöse, systemische Viruserkrankung der Hunde und anderer Karnivoren, wird als die bedrohlichste Infektionskrankheit der Marderartigen betrachtet. Empfänglich für das auslösende Morbillivirus (CDV) sind alle Karnivoren, wobei die Mortalität und Morbidität innerhalb der Familien und Arten ganz beträchtlich schwankt. Die Mortalitätsrate bei Schwarzfußilitis und Frettchen beträgt nahezu 100% und Impfung gegen die Erkrankung bei in Gefangenschaft gehaltenen Marderartigen wird dringend empfohlen. Europäische Fischotter und Asiatische Kurzkrallenotter gehören zu den am häufigsten in Europäischen Zoos gezeigten Marderartigen und leben dort in engem Kontakt zu vielen Staupe empfänglichen Arten. Leider hat sich die Impfung gegen Staupe bei Wildkarnivoren als problematisch erwiesen und nur wenige Studien wurden bisher zur Wirksamkeit der Staupeimpfstoffe bei Fischottern und anderen Wildtierarten durchgeführt. Lebendimpfstoffe, entwickelt für die Anwendung bei Haushunden, Frettchen oder Nerz, haben bei einer ganzen Reihe von Wildkarnivoren zu impfinduzierten Staupeerkrankungen geführt, auch bei nahen Verwandten der Fischotter, wie dem Frettchen, Schwarzfußiltis oder dem Europäischer Nerz. Außerdem haben sich diese Impfstoffe als nicht immer wirksam bei Mardern, einschließlich Ottern erwiesen. Sichere und wirksame Alternativen, wie inaktivierte Staupeimpfstoffe, CDV-ISCOM Impfstoff oder rekombinante Impfstoffe werden daher empfohlen. In Europa ist jedoch momentan kein zugelassener, sicherer und wirksamer Staupeimpfstoff erhältlich.

In dieser Arbeit wurde die Sicherheit und Wirksamkeit der in Europa verfügbaren Staupeimpfstoffe bei Europäischen Fischottern, Asiatischen Kurzkrallenottern und ansatzweise bei Nordamerikanischen Fischottern ermittelt. Zwei Methoden zur Antikörperbestimmung wurden verglichen. Der Referenzstandard zum Antikörpernachweis gegen Morbilliviren ist der Neutralisationstest (NT). Dieser ist zeitaufwendig und teuer. Ein kostengünstiger und schneller durchführbarer ELISA wäre sehr hilfreich. Siebzig Otter aus 19 verschiedenen Zoos und Tierparks in Deutschland und den Niederlanden, beiderlei Geschlechts und im Alter zwischen 8 Wochen und 17 Jahren wurden geimpft. Zwei Studien wurden durchgeführt. In Studie 1 wurden bisher ungeimpfte Europäische Fischotter (n=27), Asiatische Kurzkrallenotter (n=28) und Nordamerikanische Fischotter (n=4) entweder mit inaktiviertem Staupeimpfstoff, mit CDV-ISCOM Impfstoff oder mit einem von drei verschiedenen Staupe Lebendimpfstoffen geimpft. In Studie 2 wurden die Antikörper (AK) Titer Europäischer Fischotter (n=11), die bisher regelmäßig gegen Staupe geimpft wurden, vor und nach einer Boosterimpfung bestimmt.

Folgende Ergebnisse wurden ermittelt: Bei keinem der Otter wurden klinische Symptome einer Staupe Erkrankung beobachtet. Die Immunantwort auf inaktivierten Staupeimpfstoff war schwach. Zwei Impfdosen des inaktivierten Staupeimpfstoffes induzierten keine Serokonversion bei Europäischen Fischottern und Nordamerikanischen Fischottern und bei Asiatischen Kurzkrallenottern nur niedrige geometrische Mittelwerte neutralisierender AK.

CDV-ISCOM Impfstoff führte bei allen Tieren zu einer humoralen Immunantwort, hohe AK-Titer wurden aber nur im ELISA gemessen. Zieht man jedoch den Anstieg des geometrischen Mittelwertes der neutralisierenden Antikörper nach einem Jahr bei Asiatischen Kurzkrallenottern in Betracht, sowie eine eventuelle zelluläre Immunantwort und das Vorhandensein schützender AK-Titer bei einer ähnlichen Impfstudie bei diesen Otterarten, so kann nicht ausgeschlossen werden, dass der CDV-ISCOM Impfstoff eine schützende Impfantwort induziert hat. Impfung mit Lebendimpfstoff enthüllte, dass die Immunantwort vom Impfstamm abhängt. Europäische Fischotter, die mit einem an Hundenierenzellen adaptierten Impfstamm (Rockborn Typ) geimpft wurden, zeigten signifikant niedrigere AK-Titer, als Europäische Fischotter, die mit einem an Geflügelzellkulturen adaptierten Impfstamm (Onderstepoort Typ) geimpft wurden. Beide Fischotterarten entwickelten hohe neutralisierende AK-Titer nach Impfung mit einem an Geflügelzellkulturen adaptierten Impfstamm. Alter, Geschlecht, Tierart und Gewicht hatten keinen Einfluss auf die Immunantwort nach Impfung mit Staupe Lebendimpfstoff. Die drei Impfstofftypen (inaktiviert, ISCOM, lebend) zeigten einen signifikanten Unterschied in der Wirksamkeit, wobei Staupe Lebendimpfstoffe vom Onderstepoort Typ sich als am wirksamsten erwiesen. Zwischen Europäischen Fischottern und Asiatischen Kurzkrallenottern gab es keinen Unterschied in der Höhe der neutralisierenden AK-Titer nach zwei Impfdosen, jedoch einen Unterschied im zeitlichen Verlauf der Titer Entwicklung. Der Zusammenhang zwischen den zwei Methoden, Referenzstandard Neutralisationstest (NT) und dem schnellen ELISA, war nicht sehr stark. Booster Impfung bei Europäischen Fischottern mit Lebendimpfstoff (Onderstepoort Typ) induzierte einen signifikanten Anstieg der neutralisierenden AK, die bis 365 Tage nach Impfung auf einem angemessenen Level blieben.

Die Ergebnisse führten zu folgenden Schlussfolgerungen: Der Einsatz der formalin-inaktivierten Staupe Vakzine mit $Al(OH)_3$ als Adjuvant ist bei den getesteten Fischotterarten nicht sinnvoll. Staupe Lebendimpfstoffe vom Onderstepoort Typ sind wirksam bei Europäischen Fischottern und Asiatischen Kurzkrallenottern und scheinen bei diesen Arten sicher zu sein. Der experimentelle CDV-ISCOM Impfstoff ist sicher und wahrscheinlich eine wirksame Alternative. Eine jährliche Boosterimpfung wird für beide Impfstoffe empfohlen. Der in dieser Studie eingesetzte ELISA ist einsetzbar für ein Staupe AK-Screening, ist allerdings kein Ersatz für den VNT. Weitere Studien sind notwendig um die Wirkung des CDV-ISCOM Impfstoffes bei Fischottern und anderen Staupe empfänglichen Arten zu verstehen und um die Rolle des Impfstammes bei den Lebendimpfstoffen genauer zu ermitteln. Obwohl der Einsatz von Lebendimpfstoff bei Fischottern sich bisher als unproblematisch erwiesen hat, bleibt ein Restrisiko beim Einsatz eines an Hunde adaptierten Lebendimpfstoffes bei Wildkarnivoren. Die Registrierung eines sicheren und wirksamen Staupeimpfstoffes zum Einsatz bei Wildtieren in der EU wird deshalb dringend benötigt. Eine orale Vakzine wäre wünschenswert.

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APPENDIX 1:

Buffers used

1. Phosphate buffered saline solution (PBS) used for ELISA

NaCl	8.0 g/L
KCl	0.2 g/L
$Na_2HPO4*2H_2O$	1.44 g/L
KH_2PO_4	0.2 g/L
aqua destillata	1 L

The chemical substances were dissolved in aqua dest. PBS was adjusted to ph 7.4, was autoclaved and stored at 5°C.

2. ELISA buffer

400 ml PBS
\+ 0,4 g milk powder (0.1%)
\+ 0,8 g BSA (0.2%)
\+ 20 g NaCl

Until complete dissolution of all components, the ELISA buffer was stored at 5 °C.

3. Phosphate buffered saline solution (PBS) after Dulbecco and Vogt used for NLPAT

Solution A:	NaCl	80 g/L
	KCl	2.0 g/L
	$Na_2HPO_4 * 12\ H_2O$	23.7 g/L
	KH_2PO_4	2.0 g/L
Solution B:	$CaCl_2 * 2H_2O$	1.32 g/L
Solution C:	$MgCl_2 * 6H_2O$	1.0 g/L
Aqua destillata		1/L

The chemical substances of solution A were dissolved in 700 ml, in solution B and C in 100 ml each. Subsequently solution B was given slowly to solution A, the same was done with solution C. The pH was adjusted to 7.0-7.2, then 1ml /L Tween 20 (0.1%) was added. PBS was stored at 5°C.

APPENDIX 2: Otter details study 1

The table gives detailed information on the otters that were vaccinated in study 1. Eurasian otter (1), Asian small-clawed otter (2), North American river otter (3). The four age-classes are (1) cub, (2) juvenile, (3) subadult, (4) adult. The number in brackets behind the MLV vaccine indicates the vaccine used (1) Nobivac®SHP+LT, (2) Vangard®7, (3) Virbagen canis®SH_{A2}P/LT. BM = body mass, IFT= immunofluorescence test.

ID	Species	Sex	Birthdate	ageclass	BM	Zoo	Vaccine	Date of	Remarks
					(kg)			1st vaccination	
LL1	1	m	24.07.2000	4	8,65	Osnabrück	inactivated	26.01.2005	
LL2	1	m	30.08.2001	4	8,65	Osnabrück	inactivated	26.01.2005	
LL3	1	m	31.01.2003	2	8,10	Osnabrück	inactivated	26.01.2005	
LL4	1	m	31.01.2003	2	8,95	Osnabrück	inactivated	26.01.2005	
LL5	1	f	16.12.2004	1	1,45	Köln	MLV (2)	14.02.2005	moved to Otterzentrum Hankensbüttel
LL7	1	f	07.03.2000	4	5,10	Rostock	inactivated	13.04.2005	
LL8	1	f	03.07.2000	4	5,02	Rostock	inactivated	13.04.2005	
LL9	1	m	03.07.2000	4	7,22	Rostock	inactivated	13.04.2005	
LL10	1	m	24.06.2004	2	6,15	Hoyerswerda	inactivated	20.04.2005	moved to Tierpark Neumünster and Sababurg
LL11	1	f	01.07.1999	4	4,82	Hoyerswerda	inactivated	20.04.2005	moved to Tierpark Neumünster
LL12	1	f	24.06.2004	2	4,85	Hoyerswerda	inactivated	20.04.2005	moved to Tierpark Neumünster
LL13	1	m	01.10.2003	3	8,20	Hoyerswerda	inactivated	20.04.2005	
LL14	1	f	05.11.2003	3	4,70	Hoyerswerda	inactivated	20.04.2005	
LL15	1	f	01.07.2003	3	4,20	Bielefeld	ISCOM	10.05.2005	
LL16	1	f	01.07.2003	3	4,50	Bielefeld	ISCOM	10.05.2005	
LL17	1	m	02.05.1997	4	8,20	Hankensbüttel	MLV (1)	01.07.1997	
LL18	1	m	01.04.2002	4	6,20	Hankensbüttel	MLV (1)	19.04.2003	
LL19	1	m	22.08.2001	4	9,00	Hankensbüttel	MLV (1)	25.11.2001	
LL20	1	f	27.08.2001	4	6,16	Hankensbüttel	MLV (1)	29.11.2001	

ID	Species	Sex	Birthdate	Ageclass	BM	Zoo	Vaccine	Date of	Remarks
					(kg)			1st vaccination	
LL21	1	m	01.07.2003	3	7,20	Hankensbüttel	MLV (1)	20.07.2005	
LL22	1	m	28.08.1999	4	8,20	Hankensbüttel	MLV (1)	16.06.2000	
LL23	1	m	27.02.1998	4	6,90	Hankensbüttel	MLV (1)	17.08.2000	
LL24	1	f	01.05.1997	4	4,30	Hankensbüttel	MLV (1)	25.11.1997	died, 04.10.2005, necropsy, CDV negativ (IFT)
LL25	1	m	01.05.1997	4	7,50	Hankensbüttel	MLV (1)	01.02.1999	died, 27.06.2006, necropsy, CDV negativ (IFT)
LL26	1	m	06.07.1999	4	6,80	Hankensbüttel	MLV (1)	20.09.2002	
LL27	1	f	15.09.2000	4	4,90	Hankensbüttel	MLV (1)	24.04.2001	
LL28	1	m	01.09.1997	4	7,50	Hankensbüttel	MLV (1)	24.04.2001	died, 31.05.06, necropsy, CDV negativ (IFT)
LL29	1	m	17.06.2005	2	1,80	Neumünster	MLV (2)	06.10.2006	
LL30	1	f	17.08.2005	1	1,30	Neumünster	MLV (2)	06.10.2005	moved to Tierpark Ueckermünde
LL31	1	m	18.10.2003	3	7,00	Görlitz	MLV (1)	16.02.2006	
LL32	1	f	01.07.1996	4	4,30	Hanstedt	MLV (2)	23.02.2006	was found dead 10/2006, no necropsy
LL33	1	m	01.07.1996	4	6,30	Hanstedt	MLV (2)	23.03.2006	was found dead 07/2007, no necropsy
LL34	1	m	17.02.2006	1	1,20	Neumünster	MLV (2)	14.06.2006	moved to Austria
LL35	1	m	01.07.2001	4	8,00	Eindhoven/Nuenen	ISCOM	27.06.2006	
LL36	1	f	01.07.2001	4	4,00	Eindhoven/Nuenen	ISCOM	27.06.2006	
LL37	1	f	17.02.2006	1	1,30	Neumünster	MLV (1)	13.07.2006	vanished
LL38	1	f	18.05.2006	1	1,00	Neumünster	MLV (2)	03.08.2006	moved to Austria
LL39	1	f	01.06.2006	1	1,30	Neumünster	MLV (1)	01.09.2006	
Lc1	3	f	01.07.1990	4	9,00	Bad Mergentheim	inactivated	22.04.2005	was found dead 07/2006, no necropsy
Lc2	3	m	01.04.2000	4	7,60	Bad Mergentheim	inactivated	22.04.2005	
Lc3	3	f	01.03.2004	3	9,10	Gelsenkirchen	inactivated	23.05.2005	
Lc4	3	f	01.03.2004	3	8,50	Gelsenkirchen	inactivated	23.05.2005	
Ac1	2	f	07.05.2004	2	2,46	Düsseldorf	inactivated	04.01.2005	moved to Sóstó Zoo, Hungary
Ac2	2	m	07.05.2004	2	2,80	Düsseldorf	inactivated	04.01.2005	moved to Sóstó Zoo, Hungary
Ac3	2	m	14.02.2000	4	3,60	Düsseldorf	inactivated	04.01.2005	moved to Zagreb Zoo, Croatia

ID	Species	Sex	Birthdate	Ageclass	BM	Zoo	Vaccine	Date of	Remarks
					(kg)			1st vaccination	
Ac4	2	f	07.05.2004	2	2,20	Düsseldorf	inactivated	04.01.2005	moved to Sóstó Zoo, Hungary
Ac5	2	f	17.06.1995	4	3,50	Cottbus	inactivated	19.03.2005	
Ac6	2	f	03.01.2005	1	1,20	Cottbus	inactivated	18.04.2005	
Ac7	2	m	03.01.2005	1	1,30	Cottbus	inactivated	18.04.2005	
Ac8	2	f	01.04.1997	4	2,80	Cottbus	inactivated	18.04.2005	
Ac9	2	f	29.06.1992	4	2,75	Frankfurt (Main)	inactivated	17.05.2005	
Ac10	2	m	13.04.1994	4	3,40	Frankfurt (Main)	inactivated	17.05.2005	
Ac11	2	f	01.06.1998	4	2,60	Aschersleben	inactivated	22.02.2006	
Ac12	2	m	01.06.1098	4	3,20	Aschersleben	inactivated	22.02.2006	
Ac13	2	m	03.02.2006	1	1,08	Amsterdam	ISCOM	15.05.2006	moved to Randers Tropical Zoo, Denmark
Ac14	2	f	03.02.2006	1	1,07	Amsterdam	ISCOM	15.05.2006	drowned after electric shock, 04/2007, no necropsy
Ac15	2	m	03.02.2006	1	1,20	Amsterdam	ISCOM	15.05.2006	
Ac16	2	f	03.02.2006	1	1,21	Amsterdam	ISCOM	15.05.2006	
Ac17	2	f	01.06.2005	2	2,80	Neumünster	MLV (3)	14.06.2006	moved to the USA
Ac18	2	m	01.06.2005	2	3,20	Neumünster	MLV (3)	14.06.2006	moved to the USA
Ac19	2	m	01.06.2005	2	2,70	Neumünster	MLV (3)	14.06.2006	moved to the USA
Ac20	2	m	06.01.1999	4	3,10	Beekse Bergen	ISCOM	28.06.2006	
Ac21	2	f	11.08.2004	3	2,90	Beekse Bergen	ISCOM	28.06.2006	
Ac22	2	f	30.05.2002	4	2,80	Beekse Bergen	ISCOM	28.06.2006	
Ac23	2	f	11.08.2004	3	2,80	Beekse Bergen	ISCOM	08.06.2006	
Ac24	2	f	17.12.2003	4	2,40	Frankfurt (Main)	ISCOM	09.10.2006	
Ac25	2	m	08.09.2005	3	2,90	Frankfurt (Main)	ISCOM	09.10.2006	
Ac26	2	m	08.08.2006	2	1,90	Eindhoven/Nuenen	MLV (2)	05.07.2007	
Ac27	2	f	22.11.2005	3	2,40	Braunschweig	MLV (3)	19.10.2007	vanished
Ac28	2	f	24.10.2006	3	2,50	Braunschweig	MLV (3)	19.10.2007	

Appendix III: Otter and vaccination details study 2

This table gives details on the Eurasian otters vaccinated in study 2 including their vaccination history. The age classes are (3) subadult and (4) adult. BW = body mass, n.k. = not known, IFT = immunofluorescence test. All otters were housed in the Otterzentrum in Hankensbüttel.

ID	Name	Sex	Birthdate	Age class	BM	Date o	Vaccine	Remarks
					(kg)	vaccination		
LL17	Johann	m	02.05.1997	4	8,20	01.07.1997	Candivac® SHLT+P	
						07.08.1997	“	
						24.07.1998	“	
						30.11.1999	“	
						31.01.2001	“	
						09.05.2002	Nobivac® SHPPLT	
						29.10.2003	“	
						20.07.2005	Nobivac® SHP+LT	
LL18	Nico	m	03/2002	4	6,20	19.04.2003	Nobivac® SHPPiLT	
						18.07.2004	“	
						20.07.2005	Nobivac® SHP+LT	
LL19	Max	m	27.08.2001	4	9,00	29.11.2001	Canimed®SHP+L	
						10.01.2002	Canimed® SHP+LT	
						16.01.2003	Nobivac® SHPPiLT	
						24.11.2004	“	
						20.07.2005	Nobivac® SHP+LT	
LL20	Mette	f	27.08.2001	4	6,16	29.11.2001	Canimed® SHP+L	
						10.01.2002	Canidvac® SHLT+P	
						16.01.2003	Nobivac® SHPPiLT	
						01.08.2004	“	
						20.07.2005	Nobivac® SHP+LT	

ID	Name	Sex	Birthdate	Ageclass	BM	Date of	Vaccine	Remarks
					(kg)	vaccination		
LL22	Thomas	m	28.08.1999	4	8.20	16.06.2000	Canidvac® SHLT+P	
						13.08.2001	“Nobivac® ShPPiLT	
						28.01.2003	“”	
						20.07.2005	Nobivac® SHP+LT	
LL23	Kuno II	m	27.02.1998	4	6.90	17.08.2000	Candivac® SHLT+P	
						12.08.2001	“	
						12.09.2002	Nobivac® SHPPiLT	
						29.10.2003	“	
						21.07.2005	Nobivc® SHP+LT	
LL24	Kira	f	05/1997	4	4.3	25.11.1997	Canimed® SHP-L	died 04.10.05, necropsy CDV negative (IFT)
						18.12.1997	Canimed® SHP-LT	
						14.11.1998	Canestran® SHLP+T	
						30.11.1999	Candivac® SHLT+P	
						31.01.2001	“	
						11.02.2002	Canimed® SHPl-T	
						16.01.2003	Candivac® SHLT+P	
						23.02.2004	Nobivac® SHPP	
						20.07.2005	Nobivac® SHP+LT	
LL25	Robert	m	1997	4	7.5	01.02.1999	Nobivac® SHPPiLT	died 27.06.06, necropsy CDV negative (IFT)
						12.02.2000	Candivac® SHLT+P	
						23.04.2002	Canimed® SHPPi	
						29.10.2003	Nobivac® SHP-LT	
						21.07.2005	Nobivac® SHP-LT	
LL26	Oli	m	06.07.1999	4	6.80	20.09.2002	Nobivac® LT	
						29.10.2003	Nobivac® LT	
						01.09.2005	Nobivac ®SHP+LT	

ID	Name	Sex	Birthdate	Ageclass	BM	Date of	Vaccine	Remarks
					(kg)	vaccination		
LL27	Cara	f	15.09.2000	4	4.90	24.04.2001	n.k.	
						13.05.2002	Canimed® SHPPi	
						01.09.2005	Nobivac® SHP+LT	
LL28	Lukas	m	01.09.1997	4	7.50	01.02.1999	Nobivac® SHP+LT	died 31.05.06, necropsy, CDV negative (IFT)
						12.12.2000		
						31.01.2001		
						23.04.2002		
						30.10.2003		
						01.09.2005		

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PUBLICATIONS

Günther-Weigl, A. and Fröhlich, K. (2006): Staupe – Impfungen bei Ottern, ein Projekt aus dem Institut für Zoo- und Wildtierforschung, Berlin. Deutscher Wildgehege Verband (DWV) – News 1, 18-19.

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Ruff, K., Krüger, H.-H., Sewell, A.C., **Günther-Weigl, A**., Steinlechner, S. and Zentek, J. : Dietary risk factors for urate urolithiasis in Eurasian otters (*Lutra lutra*). *Submitted.*

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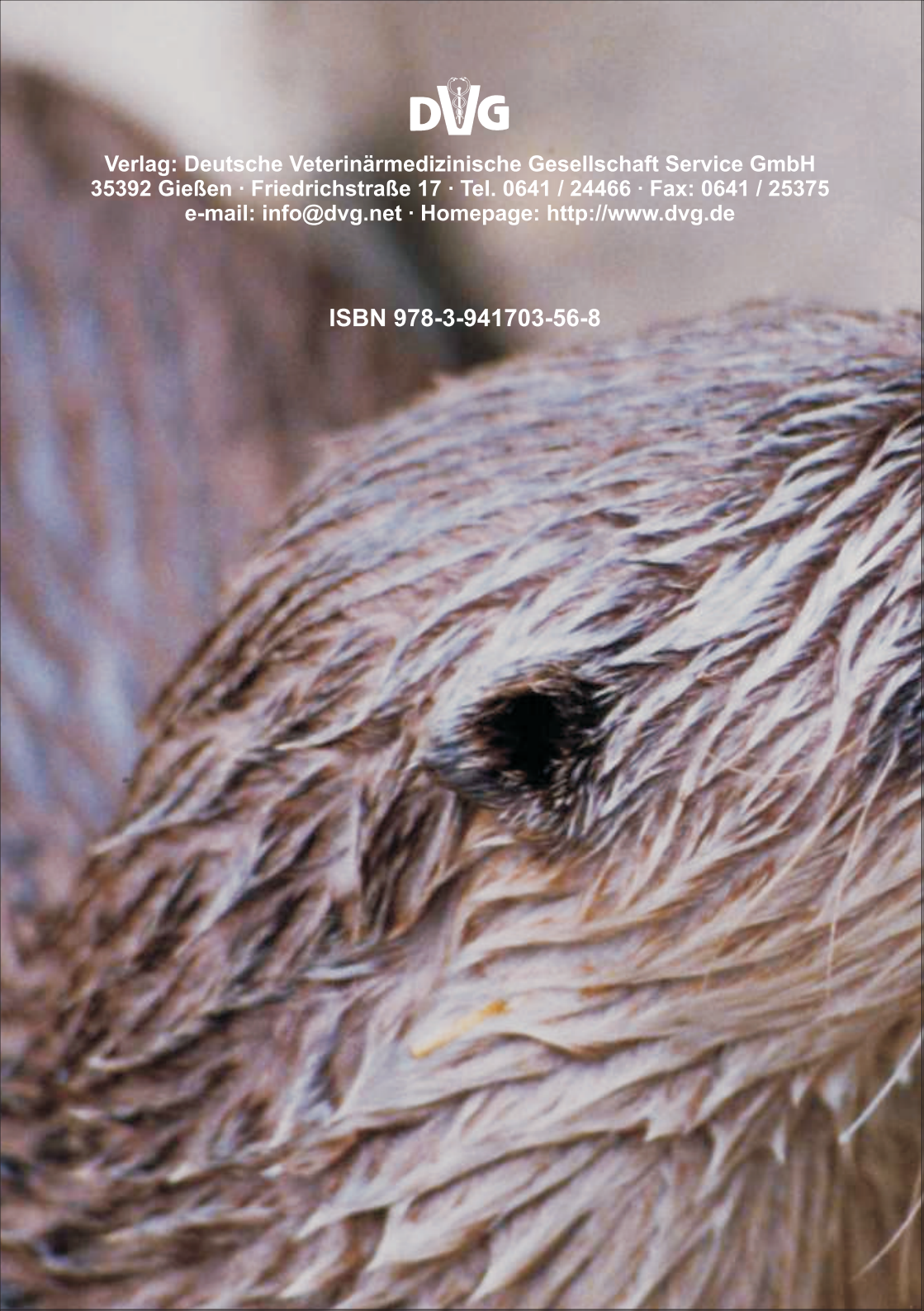
Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 28.07.2009 Astrid Günther-Weigl



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