FACULTY OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

VETERINARY MASTER'S THESIS

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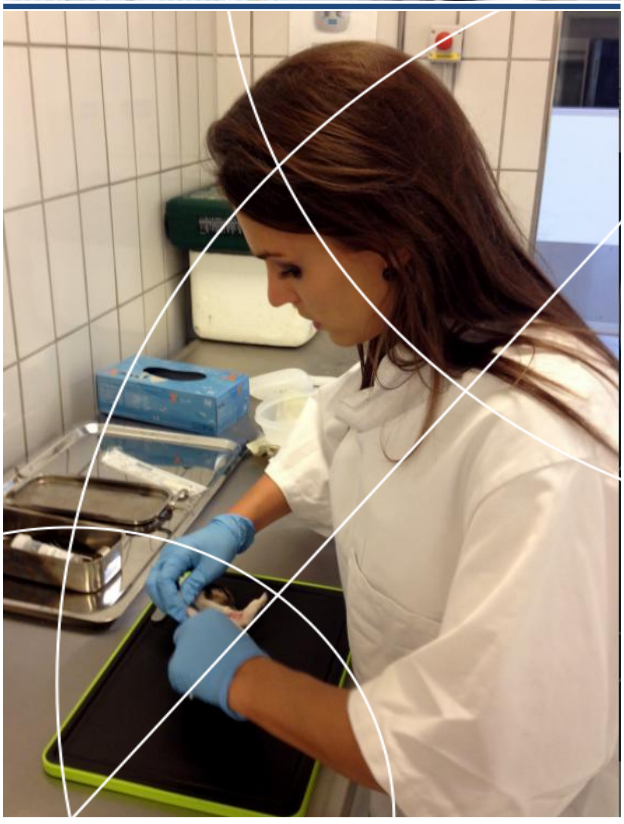
CANINE HERPESVIRUS -1 INFECTION IN NEONATAL DOGS



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ABSTRACT

Canine Herpesvirus-1 (CHV-1) causes a fatal hemorrhagic disease in neonatal dogs. Even though it has been associated with reproductive problems in female dogs, the infection in the adult dogs is usually asymptomatic. The virus is a well-known pathogen in the dog population. However, the occurrence of the infection in neonatal dogs is believed to be insignificant with regard to canine neonatal mortality.

This study aimed to determine the apparent prevalence of CHV-1 infection in dead neonatal dogs and to present the pathological findings of infection. Furthermore, risk factors and pathological findings associated with infection were analyzed by means of logistic regression analysis to investigate statistical significances.

From the cross-sectional study of 58 dead neonatal dogs, 24.5% (n = 14) of the puppies were infected. The infections were confirmed by detection of viral CHV-1 DNA of samples from the liver, spleen, lungs and kidneys using quantitative real-time Polymerase Chain Reaction (qPCR). The pathological findings varied from characteristic petechial and ecchymotic hemorrhages of several organs to nonspecific findings. In some of the puppies autolysis and congestion limited the ability to identify relevant pathological lesions. Nevertheless, the pathological findings were demonstrated to have a statistical significant association of CHV-1 infection in neonatal dogs with P values < 0.05. None of the investigated risk factors were significantly associated with CHV-1 infection.

This study indicated that CHV-1 infection had an apparent prevalence of 24.5% in the population of dead neonatal dogs. It was not possible to determine if CHV-1 was the cause of death for the infected puppies due to the lack of histopathological examination of viral replication in the organs. However, a high concentration of viral DNA and characteristic pathological lesions highly indicated that the puppies died due to the infection.

There is no available therapy for puppies with a generalized CHV-1 infection and this study suggests that breeders and veterinarians should implement prophylactic procedures including the vaccination of dams during pregnancy in order to protect neonatal dogs from infection.

RESUME

Canine Herpesvirus-1 (CHV-1) forårsager en dødelig infektion hos nyfødte hvalpe. Inficerede voksne hunde har ofte ingen symptomer, selvom infektionen er blevet forbundet med reproduktionsproblemer hos tæven. CHV-1 er en almen kendt patogen i hundepopulationen, men på trods af dette, er forekomsten af CHV-1hos nyfødte hvalpe ukendt og i forbindelse med dødeligheden blandt de nyfødte hvalpe tildeles infektionen ingen betydning.

Formålet med opgaven var, at finde den tilsyneladende prævalens af døde hvalpe smittede med CHV-1 og beskrive de patologiske forandringer forbundet med virus. Derudover blev associerede risikofaktorer og patologiske forandringer analyseret ved en logistisk regressions analyse for at se, om der var en sammenhæng mellem disse og infektionen.

Dette studie var et cross-sectional studie baseret på data fra 58 døde hvalpe. 24,5 % (n = 14) af hvalpene var smittede med CHV-1. Hvalpene blev testet positive ved fund af virus DNA fra organerne: lever, milt, lunge og nyrer ved brugen af en kvantitativ PCR analyse. De patologiske fund varierede fra karakteristiske læsioner med punktblødninger til større blødninger i flere organer. I andre hvalpe blev der ikke fundet karakteristiske læsioner, da det var svært at identificere disse på grund af stase og autolyse i organerne. Ikke desto mindre, havde de patologiske fund en statistisk korrelation med CHV-1 infektionen med *P* – værdier < 0. 05. Ingen af de undersøgte risikofaktorer havde en statistisk korrelation med infektionen.

Dette studie indikerede at den tilsyneladende prævalens af smittede hvalpe er 24,5 % i populationen af hvalpe som dør indenfor de første tre leveuger. Det var ikke muligt at afgøre om de smittede reelt døde som følge af infektionen på grund af en manglende histopatologisk undersøgelse for at afgøre om virus havde replikeret i organerne. Dog var en høj koncentration af virus DNA og karakteristiske patologiske fund indikation for, at hvalpene var døde som følge af infektionen.

Der findes inden behandling for hvalpe med en generaliseret CHV-1 infektion. Dette studie foreslår, at avlere og dyrlæger bør implementere forebyggende tiltag, herunder vaccinering af tæven under drægtigheden, for at beskytte nyfødte hvalpe mod infektionen.

PREFACE

This thesis was made as the final project in my education of Veterinary Medicine. It was performed and conducted in collaboration with the Department of Large Animal Sciences, Faculty of Health and Medical Sciences at the Department of Veterinary Reproduction and Obstetrics, University of Copenhagen. The thesis is addressed to veterinarians, students, researchers, breeders and others of interest in reproduction and management of neonatal dogs.

A special dedication and gratitude to the breeders and the participating veterinary clinics without their help and efforts, none of this work would have been possible.

I would like to thank for the generous financial support provided by Merial Norden A/S and The Danish Kennel Club. Furthermore, I would like to thank IDEXX laboratory for financing some of the laboratory tests.

A special thanks to Professor Jørgen S. Agerholm, as main academic advisor for his assistance with the postmortem examinations and the kindly and constructive guidance during the process.

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Frederiksberg, the 18th of June 2013.

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Rikke Wendt Larsen, CBR769

Frontpage photos by Rikke Wendt Larsen with purchase right to the top photo from Istockphoto.com. To the bottom: The author performing necropsy of one of the submitted puppies.

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LIST OF ABRIVIATIONS

BCS:	Body Condition Score
CHV-1:	Canine Herpesvirus – 1
CI:	Confidence interval
DKC:	Dog kidney cells
gB:	Glycoprotein B, surface protein of the Canine Herpesvirus-1.
H&E:	Hematoxylin –erosin staining
ITB:	Infectious tracheobronchitis
OR:	Odds ratio
PCR:	Polymerase Chain Reaction
qPCR:	Quantitative Polymerase Chain Reaction

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INTRODUCTION

Canine Herpesvirus - 1 (CHV-1) was first reported in the USA by Carmichael *et al.* (1965) as a cytopathogenic agent responsible for causing a generalized fatal hemorrhagic infection in neonatal dogs less than three weeks of age. In the adult dog, infection with CHV-1 is usually asymptomatic even though it has been demonstrated to cause reproductive disorders of the pregnant bitch (Hashimoto *et al.* 1982; Hashimoto *et al.* 1983). The virus has been reported in many European countries, and several serological studies indicate that CHV-1 is a well-known pathogen in the healthy dog population. In studies from Belgium, Norway, UK and the Netherlands, the prevalence of antibodies against CHV-1 varies from 40-88 % (Ronsse *et al.* 2002; Reading & Field 1998; Rijsewijk *et al.* 1999; Krogenæs *et al.* 2012).

The virus is temperature-sensitive with optimal replication at temperatures of less than 37˚ C (Greene 2012; Carmichael & Barnes 1969). During the first three weeks of life, neonatal dogs are impaired of normal temperature regulation and in addition the newborn puppies have a poorly developed immune system which makes them highly vulnerable to CHV-1 infections (Carmichael *et al.* 1969; Rootwelt *et al.* 2009: Carmichael 1970). The virus enters the bloodstream and replicates in the vascular endothelium leading to necrotizing vasculitis with secondary hemorrhages of several organs (Carmichael 1970; McGavin & Zachary 2007).

Little is known about the significance of CHV-1 as an etiological agent in canine neonatal mortality. The mortality of newborn puppies is known to be relatively high and can be related to many factors including dystocia, maternal neglect or carelessness, lack of nutrition, congenital abnormalities, cannibalism or infections (Kirkbride 2012; Indrebø *et al.* 2007).

There is no available therapy for puppies with signs of generalized CHV-1 infections and the mortality of infected litters can be high (Decaro *et al.* 2008). Specific antibodies against CHV-1 in the serum and colostrum of bitches are assumed to protect neonatal dogs from the infection (Poulet *et al.* 2001; Carmichael 1970). An inactivated vaccine has been licensed in Europe to be administered in pregnant bitches in order to provide passive maternal immunity to the puppies at whelping (EMEA 2002). However, the vaccine is not used or recommended in a standard vaccination protocol of pregnant bitches (Ronsse *et al.* 2005; Day *et al.* 2007).

To the author's knowledge there have been no previous investigations of CHV-1 infection in neonatal dogs. Other studies have investigated the causes of canine neonatal mortality in general, though CHV-1 infections and mortality due to the infections seemed to be insignificant (Gill 2001; Rota *et al.* 2007; Indrebø *et al.* 2007). The information and occurrence of the adverse effect regarding CHV-1 infection in neonatal dogs is sparse and might mask the potential of this pathogen as an important etiological agent of canine neonatal mortality. Breeders and veterinarians may need to implement prophylactical procedures including vaccination of the dams during pregnancy in order to protect neonatal dogs from infection.

AIM OF THE STUDY

The aim of this study was to present the pathological findings in neonatal dogs infected with CHV-1 and to determine the apparent prevalence of CHV-1 infection. Additionally, risk factors and pathological findings were investigated for statistically significant association with CHV-1 infection.

LIMITATIONS

The study investigated CHV-1 infection in dead neonatal dogs through postmortem examination and detection of viral DNA in selected organs. It was not possible to determine if the infection was the cause of death due to the lack of histopathological examinations. Nor was it possible to determine how the puppies had acquired the infection.

OUTLINE

The thesis is subdivided in two different parts. Part I: The literature study, which contributes with recent knowledge of CHV-1 mainly concerning infections in neonatal dogs. Furthermore, the literature study contains a section regarding canine neonatal mortality in general. Part II: The experimental part with material and methods, results and finally the discussion, conclusion and future perspectives.

PART I. – THE LITERATURE STUDY

2.1 CANINE NEONATAL MORTALITY

The mortality of newborn puppies is known to be relatively high and is considered to be a significant problem to many breeders with regards to emotional effects as well as economic losses. The neonatal period has no universally accepted definition. However, the period is usually referring to the first two to three weeks of life (Tønnessen *et al.* 2012; Indrebø *et al.* 2007). From the investigation of canine neonatal mortality in four large breeds Indrebø *et al*. (2007) found a mortality rate of 16.9 % in the neonatal period, though emphasized that other studies had found a mortality of 17-30 % within the first 8 weeks of life (Indrebø *et al.* 2007). In a study of perinatal and late neonatal mortality in the dog a mortality of 18.5 % was found from the investigation of 2574 puppies. Furthermore, Tønnesen *et al.* (2012) discovered a variation in neonatal mortality between breeds and the mortality to be depending on the size of the litter.

The immature status of the newborn puppies makes them vulnerable and depending of the intensive care from the dam within the first few weeks of life (Simpson *et al.* 2004; Indrebø *et al.* 2007). Newborn puppies have a poorly developed thermoregulation which makes them susceptible to hypothermia and due to the immature function of the kidneys they are at increased risk of dehydration (Simpson *et al.* 2004). The immature status of the immune system makes them highly vulnerable to infections (Day 2007). Further, the puppies have relatively small reserves of glycogen in the liver. In failure to suck or in lack of nutrition, the puppies may rapidly develop hypoglycemia (Simpson *et al.* 2004). Mortality in the neonatal period can be related to many factors including dystocia, maternal neglect or carelessness, lack of nutrition, congenital abnormalities, environmental conditions or infectious agents (Münnich 2008; Indrebø *et al*. 2007).

The causes of neonatal mortality have been investigated for many years in order to provide breeders and veterinarians with information and advices regarding management and care of neonatal dogs. In a study from Australia which included 475 dead puppies from 44 different breeds, the majority of the puppy losses were attributed to foetal asphyxia (Gill 2001). Others have reported that infectious diseases, mainly bacterial infections, are the second most important cause of neonatal mortality (Münnich 2008). Additionally, it has been shown that the “Fading puppy syndrome” contributes to the

neonatal losses (Gill 2001; Indrebø *et al.* 2007; Tønnesen *et al.* 2012). Breeders use the term "Fading Puppy Syndrome" for puppies that are apparently born healthy and then fails to thrive and suddenly die within the first weeks of life (Ranjan 2010). This happens despite intensive treatment and nursing care. The etiology is diverse and includes a whole range of causes as, hypothermia, mismothering, inadequate nutrition and uptake of colostrum, trauma, congenital abnormalities, low birth weight, bacterial and viral infections (Indrebø *et al.* 2007; Ranjan 2010). CHV-1 appears to be one of the important viral agents in "Fading Puppy Syndrome" (Ranjan 2010). In spite of this, the occurrences of CHV-1 infection in neonatal dogs are believed to have a relatively low prevalence with regards to canine neonatal mortality (Greene 2012; Rota *et al.* 2007; Gill 2001).

2.2 CLASSIFICATION AND PROPERTIES OF CHV-1

CHV-1 is an enveloped double stranded DNA virus belonging to the family *Herpesviridae* and the subfamily *Alphaherpesviride,* genus *Varicellovirus* (Dubovi & Maclachlan 2010). CHV-1 is phylogenetic similar to those of α- herpes viruses affecting other species but specific receptors on the cell surface causes the virus to have a restricted host range to domestic dogs or others of the *Canidae* family (Greene 2012; Nakamichi *et al.* 2000). The virus is considered to be poorly immunogenic. Neutralizing antibodies increases after infection and can remain high and detectable. However, it is assumed that antibodies to CHV-1 persist in no more than sixty days (Burr *et al.* 1996 cf. Evermann 1989).

Carmichael & Barnes (1969) studied the growth of CHV-1 in cultures of primary dog kidney cells (DKC) and in canine macrophage cultures and showed that the optimal temperature for maximal viral growth occurred at 35-36° C. The virus has affinity for epithelial cells in the mucosa of the upper airways and reproductive tract, where the temperature is optimal for virus replication (Greene 2012). Only one serotype of the canine herpesvirus has been recognized though it might be possible that other isolates or strains of CHV-1 exist with a different pathogenicity for the respiratory and genital tract (Ronsse *et al.* 2004).

The virus is inactivated at pH < 5 and >8 and by the exposure of most disinfectant, to lipid solvents and to heat above 40°C (Greene 2012). Because the virus is sensitive and quickly destroyed when exposed to environmental factors, transmission occurs by direct contact with mucosal secretions.

2.3 PATHOGENESIS AND CLINICAL SIGNS

Infections with CHV-1 can manifest itself in different ways depending on age, gender, immune system and the route of transmission as illustrated in figure 1. Infections during pregnancy, the neonatal period and in the adult dog are summarized in the following sections with aspects of the pathogenesis and clinical signs.

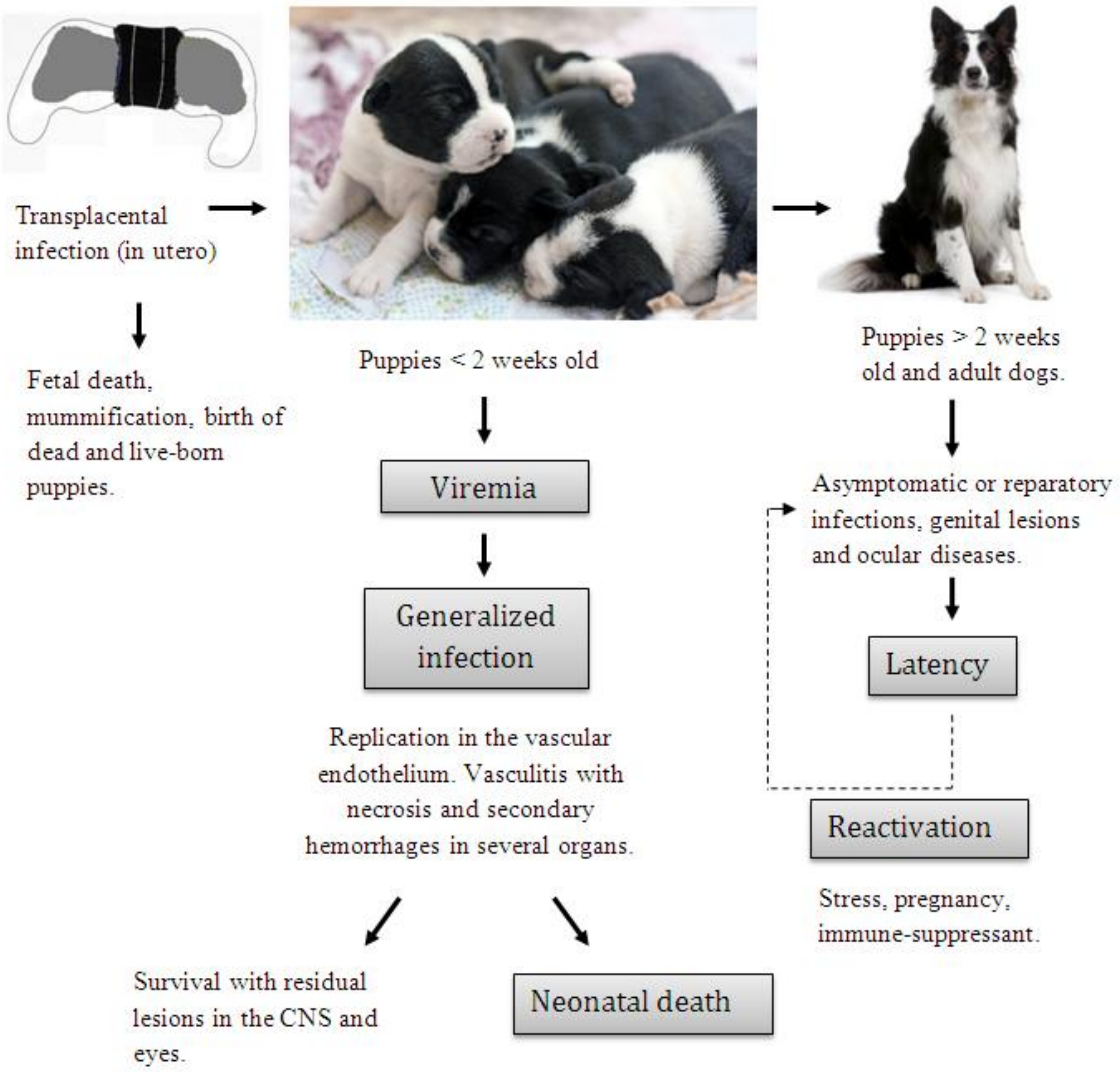

FIGURE 1. Pathogenesis and manifestation of infections with CHV-1. Note, that puppies older than two weeks are relatively unaffected by the virus whereas puppies younger than two weeks at the time of infection can develop viremia with generalized infection of several organs. (Modified figure of Harrison 2013)

2.2.1 INFECTION DURING PREGNANCY

Hashimoto *et al.* (1983) demonstrated that a virus reactivation or a primary infection of CHV-1 during pregnancy can cause transplacental transmission to the fetus. In general, virus belonging to the family *Herpesviridae,* can be transmitted transplacentally and cause placental necrotizing lesions as describes from other mammals like humans, horses and cattle (Hashimoto *et al.* 1979; Dubovi & Maclachlan 2010). As for other mammals and dogs, the outcome of the pregnancy depends on the stage of gestation at which infection occurs. Experimental intravenous virus administration to pregnant bitches from second trimester to end of gestation has been published (Hashimoto *et al.* 1982; Hashimoto *et al.* 1983). Intravenous inoculation of pregnant bitches with CHV-1 on day 30 and 40 of gestation resulted in transplacentral infections of the fetus with presence of fetal death, abortion, mummification, premature birth and birth of dead and live-born puppies. One bitch inoculated at day 30, findings of dead and suspected mummified fetuses and three unaffected live-born puppies were obtained by cesarean section. The three live-born puppies from this litter remained clinically normal during the first 14-days of life and at postmortem no virus could be recovered from the organs by viral isolation (Hashimoto *et al.* 1983). Live puppies (n = 31) and two stillborn puppies were born, when inoculating seven pregnant bitches at the end of gestation at day 48 – 53. The majority (n = 26) of the puppies were weak and died within two weeks with symptoms of systemic CHV-1 infections and five puppies remained clinically healthy with no microscopic lesions or virus isolation that could indicate a CHV-1 infection (Hashimoto *et al.* 1982). An interesting finding in the two experimental studies was the occurrence of unaffected puppies (with no signs of systemic CHV-1 and with no viral isolation at postmortem) in litters where the majority of the puppies were apparently infected by CHV-1. The exact mechanism of this occurrence could not be clarified by the authors. However, they compared the transplacental infection by other viruses of the family *Herpesviridae,* which tend to vary with the stage of gestation and the degree of placental development, infection in utero being restricted to only some fetuses and in some instants to the amount of virus to which the fetuses are exposed (Hashimoto *et al.* 1983).

2.2.2 INFECTION DURING THE NEONATAL PERIOD

Newborn puppies may acquire the infection in utero or from passage through the birth canal but more commonly, the puppies are suspected to be infected from oronasal secretion from the dam or by infected littermates and surrounding dogs (Rootwelt *et al.* 2009; Greene 2012). Appel *et al.* (1969) pointed out that puppies, older than two weeks, at the time of infection, are relatively unaffected by the virus and the infection is generally associated with localized infections in the upper airways.

The incubation period varies from four to ten days and most of the affected puppies are less than three weeks old when signs of illness occur. The mortality of the litter can be high and may reach a mortality rate up to 100% (Decaro *et al.* 2008). Clinical signs may be presented as anorexia, loss of interest in nursing, abdominal pain, vocalization, opisthotonus, nasal discharge, sneezing and soft feces. However, sometimes the puppies present no noticeable symptoms (Carmichael *et al.* 1965; Poulet *et al*. 2001; Rootwelt *et al.* 2009). Carmichael (1970) summarized in an article that after oronasal inoculation of puppies, the primary site for virus replication was the nasal epithelium and the tonsils. After three to four days, the virus enters the bloodstream resulting in a leukocyte-associated viremia probably through the uptake of macrophages. The virus spreads through the blood and replicates in vascular endothelium lining small blood vessels, leading to necrotizing vasculitis with secondary diffuse hemorrhage in several organs including the kidneys, adrenal glands, liver, spleen and lungs (Poulet *et al.* 2001; Wright & Cornwell 1968). In a study by Percy *et al.* (1968) where puppies were naturally or experimentally exposed to CHV-1 they demonstrated that viremia resulted in foci of edema, neuronal degeneration and neutrophil infiltration of the retina. In the same study, CHV-1 affected the brain causing a non-suppurative meningoencephalitis, probably through spread from the trigeminal ganglion.

Until the third week of life, the neonatal puppies are impaired of normal temperature regulation. The rectal temperature is normally 1-1.5°C below the temperature of adult dogs, which is optimal for CHV-1 replication (Greene 2012). In a study with normally resistant four to eight week old puppies, Carmichael *et al.* (1969) reported, that when they artificially lowered the body temperature of the puppies after intraperitoneal inoculation they developed microscopic lesions and viral growth in various tissues that could not be demonstrated in inoculated littermates kept at normal temperature. In

addition to having a reduced capacity for temperature regulation, newborn puppies have a poorly developed immune system which makes them incapable of making a febrile and inflammatory response before 6 – 12 weeks of age (Day 2007). It is suggested, that temperature regulation together with a poorly developed immune system make the puppies highly vulnerable to CHV-1 infections (Carmichael *et al.* 1969; Rootwelt *et al.* 2009; Carmichael 1970).

Antibodies against CHV-1 in the serum and colostrum of seropositive bitches are assumed to reduce the risk of reproductive disorders and to protect puppies from developing the fatal systemic infection (Carmichael 1970; Poulet *et al.* 2001). Puppies nursed from seronegative bitches or from bitches with a low level of immunoglobulin may possibly develop the fatal generalized disease. Puppies nursed by seropositive bitches with a high level of immunoglobulin become infected, however with the absence of clinical illness and limited virus recovered from the oropharyngeal region (Carmichael 1970; Huxsoll & Hemelt 1970). With the presence of protective antibodies in the serum of infected bitches it is supposed, that naturally infected dams that give birth to diseased puppies will usually give birth to normal litters on subsequent pregnancies (Huxsoll & Hemelt 1970; Evermann *et al.* 2011).

Puppies that survive the generalized infection with CHV-1 are likely to have residual lesions in the CNS, eyes, lungs and kidneys (Percy *et al.* 1971; Percy *et al.* 1968) and may develop neurological signs or blindness as adult (Greene 2012; Percy *et al. 1*971).

2.4 DISEASE CAUSED BY CHV-1 IN THE ADULT DOG

2.4.1 INFECTION OF THE RESPIRATORY TRACT

CHV-1 has been associated with the multifactorial respiratory disease, infectious tracheobronchitis (ITB) also known as "Kennel Cough". Appel *et al.* (1969) inoculated 5 to 12 weeks old puppies, by the oral-nasal route with strain of CHV-1 and demonstrated virus replication in the epithelial tissue of the upper respiratory tract, causing mild clinical symptoms of rhinitis and pharyngitis. Symptoms of ITP are related to an unproductive forceful coughing and were investigated by Erles *et al.* (2004) in a two year longitudinal study of shelter dogs. Coughing was regularly observed and samples from the trachea and lungs revealed CHV-1 but also other viral agents. Multiple infectious agents, as Canine Parainfluenza Virus and *Bordetella bronchiseptica* are assumed to be frequently involved in the induction of ITB (Erles *et al.* 2004; Evermann *et al.* 2011) and the role of CHV-1 in the etiology of ITB remain controversial and is still being assessed (Evermann *et al.* 2011).

2.4.2 GENITAL LESIONS

CHV-1 replicates in the cooler temperature of the mucous membrane of the upper respiratory tract and the outer genitals. Lymphoid nodules and petechial hemorrhages have been reported on the penis and prepuce and in the vagina, when inoculated intragenitally (Hill & Maré 1974). Genital lesions may cause the virus to be transmitted during copulation. However, genital lesions in bitches are believed to be most important as a risk of transmission to puppies during birth (Hill & Maré 1974; Rootwelt *et al.* 2009).

2.4.3 OCULAR DISEASE

Retinal lesions caused by CHV-1 infections have been demonstrated in neonatal puppies (Percy *et al.* 1968) and in contrast, ocular lesions in the adult dog are typically restricted to the cornea, conjunctiva and eyelid (Evermann *et al.* 2011). In experimental topical ocular inoculation of eight dogs, Ledbetter *et al.* (2009) induced bilateral conjunctivitis in all of the dogs, followed by latency. After eight months, the virus was reactivated by prednisolone and lesions were characterized by conjunctivitis and a single dog with superficial corneal ulceration.

2.5 LATENCY

A typical characteristic of virus belonging to the family of *Herpesviridae* is the ability to cause lifelong latent infections (Dubovi & Maclachlan 2010). To determine cell types and sites of CHV-1 latency, Miyoshi *et al.* (1999) experimentally inoculated eight seronegative adult dogs by the intranasal (n = 2), intranasal and intervenously (n = 3) or intravaginal (n = 3) routes with CHV-1. Using the polymerase chain reaction (PCR) they were able to detect DNA in the trigeminal ganglia, irrespective to inoculation route. The retropharyngeal lymph nodes were another important site of latency, since viral DNA was detected in 7 of 8 dogs. Latency was also detected in the lumbosacral ganglia, but only in 4 of 8 dogs. Burr *et al.* (1996) used PCR in detection of CHV-1 in twelve key sites that had been associated with latency for other herpesviruses in 12 adult dogs that had been euthanized for various reasons. Viral DNA was detected in 9 of 12 dogs. The tissues most commonly affected were the lumbosacral ganglia, tonsils, liver and the parotid salivary glands.

Abortions and stillbirths could be associated with viral localization in the lumbosacral ganglia as the route of transmission from the reproductive tract or transplacental. However, more frequently it seems that the virus is detected in tissues related to the oronasal mucosa which could indicate that more commonly the route of transmission is from oronasal secretion. During latency, it seems that CHV-1 "hides" in the ganglionic and lymphoid tissues of the oronasal and genital mucosa (Figure 2), since none of the above mentioned studies were able to detect viral DNA in the blood (Burr *et al.* 1996; Miyoshi *et al.* 1999).

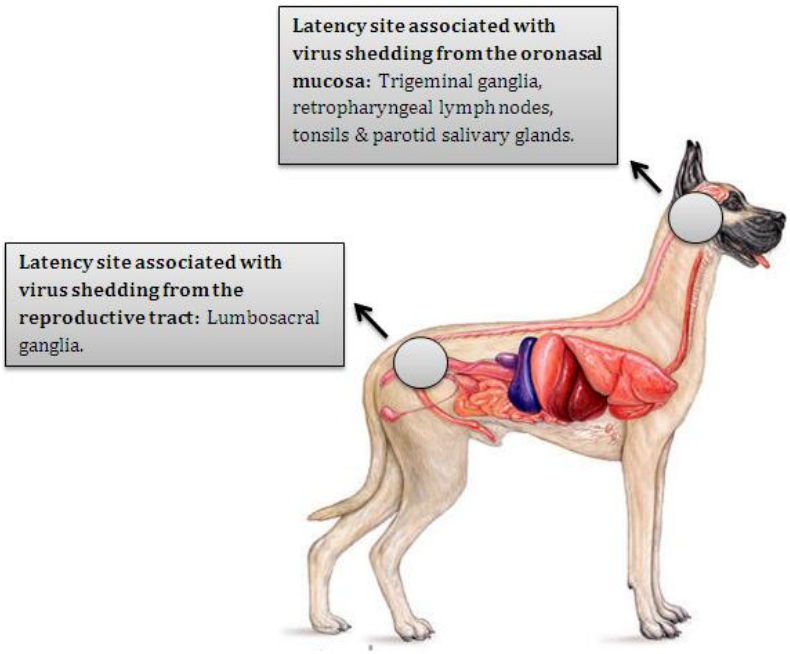

FIGURE 2. Localization of CHV-1 at latency in the adult dog. The virus "hides" in lymphoid tissue and nerve ganglia of the oronasal and genital mucosa during latency (Modified figure of O'keefe 2013).

2.6 REACTIVATION AND VIRAL SHEDDING

Reactivation occurs sporadically and is believed to be associated with stress, pregnancy and by administration of immunosuppressive drugs (Greene 2012). Reactivation of latent CHV-1 was demonstrated in prednisolone treated bitches with a history of reproduction disorders induced by the virus. The dams were given high doses of prednisolone (600 mg) for five consecutive days and reactivation was confirmed in 4 of 5 bitches (Okuda *et al.* 1993). The authors did not observe any clinical signs in the bitches but infectious CHV-1 was recovered from the nasal, oral, vaginal, and ocular mucosa from one to three weeks after initiation of treatment (Okuda *et al.* 1993). Viral shedding after reactivation is assumed only to last a few days while viral shedding during primary infection are prolonged and associated with higher viral titers than a recurrent infection (Ronsse *et al.* 2005; Okuda *et al.* 1993).

Difficulty in predicting reactivation and antibody patterns is emphasized in a study by Ronsse *et al.* (2005) who followed 27 breeding bitches during one reproductive cycle for the detection of viral DNA and specific antibodies. All the initially seronegative dogs seroconverted during the investigated time. However, 40% of the seropositive dogs became seronegative on one or two occasions. Furthermore, two types of antibody patterns were found in bitches with reduced fertility and reproductive disorders. Some had moderate to strongly positive titers throughout the reproductive cycle, and others had decreased levels of antibodies in early and late diestrous (Ronsse *et al.* 2005). When comparing mean antibody titers between mated bitches with and without reproductive disorders, there was no significant difference ($p < 0.13$). Although, figure 3 shows a tendency towards higher titers in estrus and early dioestrus in bitches without reproductive disorders (Ronsse *et al.* 2005). This could indicate a protective aspect of antibodies in serum of pregnant dams in the way of reducing the risk of reproductive disorders.

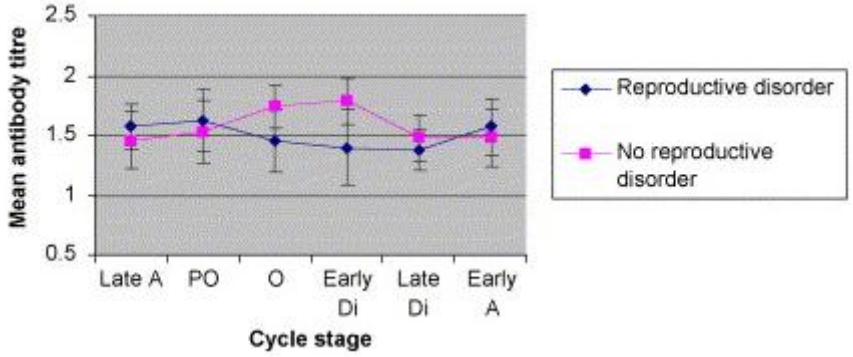

FIGURE 3. Mean antibody titers +/- S.E.M expressed as $\log_{10}$ values depending on the occurrence of reproductive disorders. No significant differences were observed (Ronsse *et al.* 2005).

2.7 DIAGNOSIS

Information obtained from the clinical history of the puppies compared with high mortality of the litter during the neonatal period, usually gives the veterinarian reason to suspect a CHV-1 infection. In order to determine CHV-1 infections, diagnosis must be based on more specific pathological and histopathological findings and detection of viral DNA in the organs of stillborn or dead neonatal puppies.

2.7.1 POSTMORTEM FINDINGS

Postmortem examination is important in the diagnostic of CHV-1. The general appearances of the puppies are often normal, but sometimes underweight (Poulet *et al.* 2001). Characteristic macroscopic lesions include petechial and ecchymotic hemorrhage and generalized enlargement of organs, especially in the kidneys, liver, spleen and small intestine, as illustrated in figure 4 (Carmichael *et al.* 1965; Wright & Cornwell 1968; Poulet *et al.* 2001). Especially the mottling, heterogeneous appearance of the kidneys (Picture B in figure 4) due to an acute viral-induced vasculitis with necrosis and secondary hemorrhage (McGavin & Zachary 2007) is believed to be a grossly characteristic of this disease (McGavin & Zachary 2007; Kirsbride 2012). The lungs fail to collapse with presence of pulmonary edema. Further, petechial to ecchymotic hemorrhages are scattered throughout the surface seen as focal greyish and reddish areas. Enlargement of lymph nodes (Carmichael *et al*. 1965) and signs of diffuse hemorrhagic meningoencephalitis and retinal lesions have been reported in puppies which were either naturally or experimentally exposed to CHV-1 (Percy *et al.* 1968).

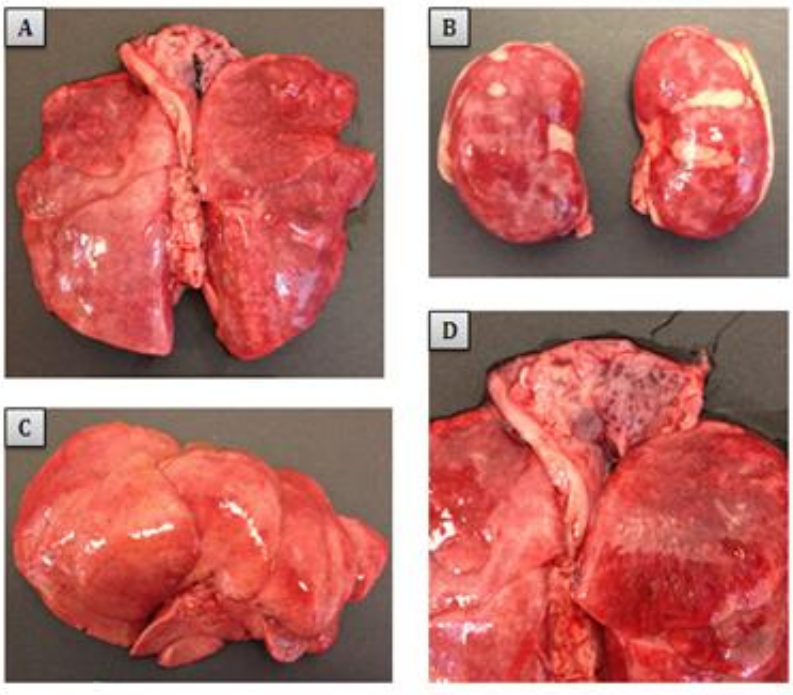

FIGURE 4. Organs from a 13 days old puppy that spontaneously died of an infection with CHV-1. **A: Lungs**, presented with edema and a heterogeneous appearance. Petechial to ecchymotic hemorrhages seen as reddish and greyish areas scattered throughout the surface. **B: Kidneys,** with petechial to eccymotic hemorrhages. **C: Liver**, was enlarged and with petechial bleedings. **D: Thymus**, presented with petechial bleedings.

2.7.2 HISTOPATHOLOGICAL FINDINGS

When staining formalin fixated tissues with a hematoxylin-eosin staining (H&E), histopathological findings of naturally or experimentally infected neonatal puppies are characterized by disseminated foci of necrosis with peripheral hemorrhages in the kidneys, lung, spleen, small intestines and the brain (Carmichael *et al.* 1965; Love & Huxtable 1976; Poulet *et al.* 2001). Carmichael *et al.* (1965) reported, that a slight cellular infiltration can be seen, but in general there are rarely any inflammatory reactions in the necrotic areas. Synthesis of viral DNA and nucleocapsids occurs within the host cell nucleus (Rootwelt *et al.* 2009). The replication is rapid and highly destructive and gives rise to intranuclear eosinophilic inclusion bodies in the periphery of the necrotic foci which are of diagnostic value (Greene 2012).

2.7.3 DETECTION OF VIRAL DNA

Viral DNA from CHV-1 can be detected by immunologic techniques, virus isolation, electron microscopy and PCR (Greene 2012). The determination and identification of CHV-1 infections has previously relied upon either virus isolation from tissues or swabs or from serological studies of antibodies. Successfully use of PCR has made this method a more common and sensitive diagnostic method in detection of viral DNA from the CHV-1 genome and especially in detection of latent infections (Burr *et al.* 1996; Decaro *et al.* 2010).

2.8 THERAPY

Symptomatic treatment for respiratory, ocular disease or genital lesions may be needed in adult dogs; otherwise no treatment is necessary as the virus is self-limiting (Evermann *et al.* 2011). Treatment with high doses antiviral drugs such as acyclovir is recommended with effective results in human neonatal herpes (Kimberlin *et al.* 2001). Successfully use of acyclovir in therapy and management of neonatal puppies infected with CHV-1 is described in a case report from California (Davidson *et al.* 2003). However, signs of toxicity have been reported in dogs from accidental ingestion of acyclovir with doses of 40 mg/kg (Richardson 2000). The pharmacokinetics, the bioavailability and the effective dose for CHV-1 in dogs are currently unknown and needs further investigation before using anti-herpesviral drugs in therapy of neonatal dogs infected with CHV-1 (Evermann *et al.* 2011). Intraperitoneal injection of 1 – 2 ml of immune sera obtained from seropositive dogs or an elevation of the puppies body temperature to reduce viral replication, seems to lower the mortality of those puppies where a generalized infection has not yet been manifested (Greene 2012). In general, therapy for neonatal dogs with signs of generalized CHV-1 infection is limited and with poor prognosis.

2.9 VACCINATION AND PROPHYLACTIC PROCEDURES

An inactivated vaccine is licensed in Europe (Eurican® Herpes 205, Merial, France) which contains a specific surface protein (gB – glycoprotein) from CHV-1 (EMEA 2002). The vaccine is administrated subcutaneously to pregnant bitches in a two dose regimen, to ensure a satisfactory neutralizing antibody level at the time of whelping. First vaccination should be at estrus or seven to ten days after mating, and second vaccination one to two weeks before whelping (EMEA 2002). Vaccination provides passive maternal immunity to the puppies, when absorbing IgG from the colostrum and milk within the first 12-36 hours of life (EMEA 2002).

Poulet *et al.* (2001) demonstrated the protection of puppies against CHV-1 by vaccinating pregnant bitches. Puppies from vaccinated and unvaccinated bitches were challenged oronasally three days after birth with a virulent CHV-1. The majority of puppies from the unvaccinated bitches died from generalized infections between 6 and 14 days after challenge. None of the puppies in the vaccinated group died from CHV-1 infection. Since little is known about the prevalence or significance of CHV-1 as an

etiological agent of the mortality in neonatal dogs, the vaccine is not used or recommended in a standard vaccination protocol for pregnant bitches (Day *et al.* 2007; Ronsse *et al.* 2005).

Vaccination should be supplemented with prophylactic procedures to further reduce the risk of CHV-1 infections in neonatal dogs. Ronsse *et al.* (2004) studied the factors that influenced antibody levels and consequently the risk of CHV-1 infections in kennel dogs. The primary contributing risk factors were the size of the kennels, hygiene and kennel cough. Kennels with a large number of dogs (> 6 dogs) and in addition a poor hygiene had high antibody levels. Further, dogs with a history of outbreaks with kennel cough had a significantly higher antibody titer than dogs without such history (Ronsse *et al.* 2004).

In order to protect neonatal dogs from infection a suggestion of a prophylactic regimen in larger kennels, could be to improve hygiene and isolation of the dam and puppies (to lower the risk of CHV-1 infections from surrounding dogs). Additionally, good environmental conditions to ensure an elevation of the body temperature, may provide some protection to uninfected puppies as the virus replication is reduced at higher temperatures (Carmichael 1970). Furthermore, colostrum intake within the first few hours of life is essential to the puppies in receiving maternal antibodies as protection against infections.

PART II. – THE EXPERIMENTAL STUDY

3.1 MATERIAL AND METHODS

3.1.1 THE STUDY DESIGN AND INCLUSION CRITERIA

The study was a cross-sectional study which proceeded from September 2012 to April 2013 with collection and necropsy of death neonatal puppies at the Department of Large Animal Sciences, Faculty of Health and Medical Sciences at the Department of Veterinary Reproduction and Obstetrics, University of Copenhagen. Puppies that spontaneously died or were euthanized less than three weeks of age were included in this study. Furthermore, puppies from cesarean sections and stillborn puppies were included because of the presumption and possibility of transplacental transmission of the virus.

3.1.2 COLLECTION OF DATA

In September 2012 posters and brochures with information about CHV-1 and the purpose of the thesis, were sent to 300 randomly selected veterinary clinics in Denmark. In order to reach potential breeders and owners with upcoming litters, a homepage on Facebook was created “Herpesvirus hos nyfødte hvalpe”, where breeders could ask questions about the thesis.

Breeders or veterinary clinics were requested to store dead puppies in the refrigerator (or at max. 5˚ C) before sending them by postal service to the department of Veterinary Reproduction and Obstetrics. In addition to keep undesirable fluid from leaking the package and in order to preserve and protect the puppies at transportation, breeders and veterinarians were requested to enfold the puppies in towels or newspapers and transport them in a solid cardboard box.

The breeders were requested to fill out a questionnaire with general questions about the bitch, the household and the submitted puppy or puppies (Appendix III).

The time between death and postmortem examination varied from hours to a number of days. Some puppies were examined by the veterinarians and medical treatment and supplementary feeding was tried for therapy before the onset of dead.

3.1.3 POPULATION

In total, 58 puppies were included in the study from 37 litters and 26 different breeds. Unfortunately, the owner from puppy no. 23 was unknown and the puppy was only partly included in the study. The puppies came from different regions of Denmark as illustrated in figure 5.

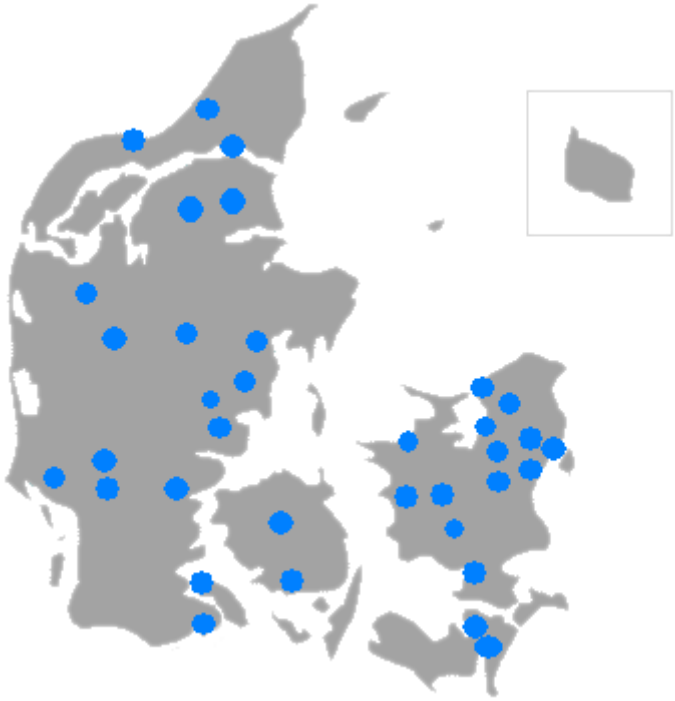

FIGURE 5. Illustration of the different regions of Denmark in which, the submitted puppies came from. Note that the population of the submitted puppies was evenly distributed throughout the regions of Denmark.

3.1.4 THE POSTMORTEM EXAMINATION

The general appearance of the puppies was examined. The puppies were sexually identified, weighted and examined for visible hemorrhages on the skin and mucosal membranes. Congenital deformities were noted as well as an estimation of the body condition score (over-/underweight/normal).

An incision through the axially and the groin area was made to place the puppy in dorsal recumbency.

The thorax was opened with an incision through the *manubrium sterni* and with a forceps holding on to the sternum, while incisions along the costal cartilage attachment

exposed the organs of thorax. The skin along the thorax was removed from the body and the subcutaneous tissue was examined for evidence of bruising, hemorrhage or edema (Figure 6).

FIGURE 6. The postmortem examination. To the left: The author is performing a postmortem examination of puppy no. 42 to the right. To the right: Presentation of the organs after removal of the sternum and opening of the abdominal cavity.

The heart, lungs and the thymus were examined for pathology and the ribs were examined for evidence of fractures.

The abdominal cavity was opened by cutting through the abdominal wall with a scissor from the *processus xiphoideus*, cranially along the rib curvature and caudally to the groin as illustrated in figure 6. The abdominal organs were examined for pathology and the stomach was examined for the presence of milk.

The brain was exposed and examined by removing the frontal and parietal bones.

3.1.5 VIRAL DETECTION OF CHV-1 USING PCR

Samples from liver, lungs, spleen and kidneys were collected for viral detection of CHV-1 DNA since these organs earlier have been shown to have the highest CHV-titers (Decaro *et al.* 2010). The organs were analyzed by quantitative real-time PCR (qPCR) at IDEXX laboratories in Germany. The samples were stored in a plastic tube at – 20 ˚C prior to the shipment to the laboratory on dry ice.

Tissues from the liver/ lung and spleen/kidney were pooled in two different tubes. Equal amounts of two tissue samples (10 mg) were taken out from each organ to make a total volume of 20 mg in the pooled sample. From puppy no. 23 and no. 24, one organ was missing and in puppy no. 25 two organs were missing and the pooled samples represented equal amounts of the remaining organs to make a total volume of 20 mg in the pooled samples. All of the internal organs from puppy no. 14 were missing and PCR was not performed. Real-time PCR at IDEXX Vet Met Lab was performed using the LightCycler 480 system (Roche) with proprietary forward and reverse primers and a hydrolysis probe. Target gene for the detection of CHV-1 was the DNA polymerase gene. Real-time PCR was run with five quality controls, including a 1) PCR-positive control, 2) PCR negative control, 3) negative extraction controls, 4) internal positive control (IPC) spiked into the lysis solution to monitor the nucleic acid extraction efficiency and presence or absence of inhibitory substance and an 5) environmental contamination monitoring control (Balzer 2013). By adding a fluorescent probe to the samples, amplification and quantification occurred simultaneously as the fluorescent detector measured the DNA copies through each thermal cycle. In a logarithmic scale a threshold for detection of DNA was set by plotting the fluorescence against the number of cycles. The cycle number at which the fluorescence signal from the amplification of the DNA exceeds the threshold is called the threshold cycle (C_t) or the crossing point (C_p) (Brookman-Amissah *et al.* 2012). Samples were considered positive if the C_t/C_p exceeded the threshold within 40 repeated cycles. Only samples with a C_t/C_p close to the threshold with a value of > 40 were repeated. If the value was the same in repeated assays it was considered positive, though corresponding to a small amount of viral DNA (Balzer 2013).

3.3 POST PROCESSING

Breeders and veterinarians were contacted by telephone or e-mail at the end of the study with results from the necropsy and PCR regarding their submitted puppy or puppies. The breeders were requested to inform about the total mortality of puppies during the neonatal period from the litter of the submitted puppy or puppies. Further, the breeders were questioned about the age of the bitch at the time of parturition in order to investigate if the age had a statistical association with CHV-1 infection in neonatal dogs. The completed questionnaire was evaluated and corrected for errors.

3.4 STATISTICS

All data were analyzed using Microsoft Excel 2010 and R 3.0.0 statistical software programming (R Core Team, 2013). Risk factors and pathological findings were screened for association with CHV-1 infected puppies by means of a univariate mixed-effect logistic regression analysis with "bitch"[1] as random effect. Results were given as *P* – values and odds ratio (OR) with 95 % confidence interval (CI). Some of the categorical variable and class descriptions were formulated on the basis of literature from Ronsse *et al.* (2004) where several risk factors were screened for affecting CHV-1 antibody titers in a dog population. Others were formulated on the basis of current literature and studies regarding CHV-1 infections (Table 1). Risk factors (Table 1) or pathological findings (Table 2) were considered significantly associated with the CHV-1 infection if ($P < 0.05$).

[1] "Bitch" was set as random effect because some of the submitted puppies came from the same bitch.

TABLE 1

Risk factors investigated for significant association with CHV-1 infection

Risk factor	*N* classes	Class description (*N* dogs per class)	*N* dams/litters	*N* puppies
Age of the bitch	4	*Age groups:* **1**: 0 - ≤ 2 years old (5) **2**: > 2 - ≤ 4 years old (19) **3**: > 4 - ≤ 6 years old (10) **4**: > 6 - ≤ 8 years old (2)	36	
Kennel size	2	*Kennel size groups:* **1**: < 6 dogs (29) **2**: > 6 dogs (7)	36	
Kennel cough	2	*Did the dam had a history of kennel cough?:* **Yes** (7) **No** (29)	36	
Mortality > 80 %	2	*Mortality > 80 % of the littersize:* **Yes**: ≥ 80 % (9) **No**: < 80 % (27)	36	
Number of previous litters	3	*Number of previous litters, groups:* **0**: (19) **1**: (9) **> 1**: (8)	36	
Reproduction disorders	2	*History of reproduction disorders:* **Yes** (7) **No** (29)	36	
Vaccinated with Eurican® Herpes 205	2	*Was the dam vaccinated:* **Yes** (1) **No** (35)	36	
Stillborn	2	Was the puppy stillborn: **Yes** (22) **No** (36)		58
Clinical signs	2	*Symptoms groups:* **Yes**: If > two symptoms (11) **No**: If < two symptoms (46)		57 *
Body condition score (BCS)	3	*Groups:* **1**: Underweight (23) **2**: Normal: (33) **3**: Overweight (2)		58

*Only 57 puppies is represented in this category because of the missing data from puppy no. 23

TABLE 2

Association between lesions and CHV-1 infection

Risk factor	*N* classes	Class description (*N* dogs per class)	*N* puppies*
Organomegaly**	2	Present: **Yes** (7) **No** (7)	14
Pulmonic edema	2	Present: **Yes** (6) **No** (8)	14
*Lesions with hemorrhages****			
In the kidneys	2	Present: **Yes** (5) **No** (9)	14
In the lungs	2	Present: **Yes** (5) **No** (9)	14
In the liver	2	Present: **Yes** (4) **No** (10)	14
In the small intestines	2	Present: **Yes** (4) **No** (10)	14
In the spleen	2	Present: **Yes** (3) **No** (11)	14

* *N* puppies represent the number of puppies with CHV-1 infection.

The total number of puppies in the study was 58.

* Organomegaly in the liver, kidneys, spleen and/or the thymus

** Hemorrhages characterized as either petechial or ecchymotic

4. RESULTS

All data, available from the questionnaire, the postmortem examination and PCR results with specified C_t/C_p values are summarized in Appendix I. The aim of the study was to present the pathological findings in neonatal dogs with CHV-1 infection and to investigate if associated risk factors and pathological findings had statistical significance of the infection. This section will outline and focus on the puppies confirmed positive of CHV-1 infection by PCR. Further, an overview of the mortality of the submitted puppies will be highlighted in order to calculate the apparent prevalence of CHV-1 infection

4.1 MORTALITY OF THE SUBMITTED PUPPIES

From the population of puppies that died during the neonatal period (n = 99, Appendix I), 58 puppies were represented in the study. Summarized data from the submitted puppies are presented in Table 3. 22 puppies were stillborn and 36 died or were euthanized during the first three weeks of life. Six of the puppies were euthanized with pentobarbital in the peritoneum or the heart. The majority (n= 46) representing 80.7 % of the submitted puppies, were either stillborn (n = 22) or died within the first week of life (n = 24). Of the submitted puppies, 35 were females and 23 were males.

TABLE 3

Overview of the mortality of the submitted puppies	*N* puppies	%
Total number of puppies	58	
Stillborn	22	**37,9**
Live-born puppies	36	**62,1**
Euthanized	6	
Died within the first week	46	**80,7**
Died (> 7 days old)	11	**19,3**
Females	35	
Males	23	

4.2 THE APPARENT PREVALENCE OF CHV-1 INFECTION

In total, 58 puppies were included in the study and 14 puppies were positive of CHV-1 infection confirmed by detection of CHV-1 DNA by PCR. The PCR was performed on 57 of the submitted puppies as the organs of puppy no. 14 were missing (Appendix I). The prevalence of CHV-1 infection from the submitted puppies was 24.5% as illustrated in Figure 7.

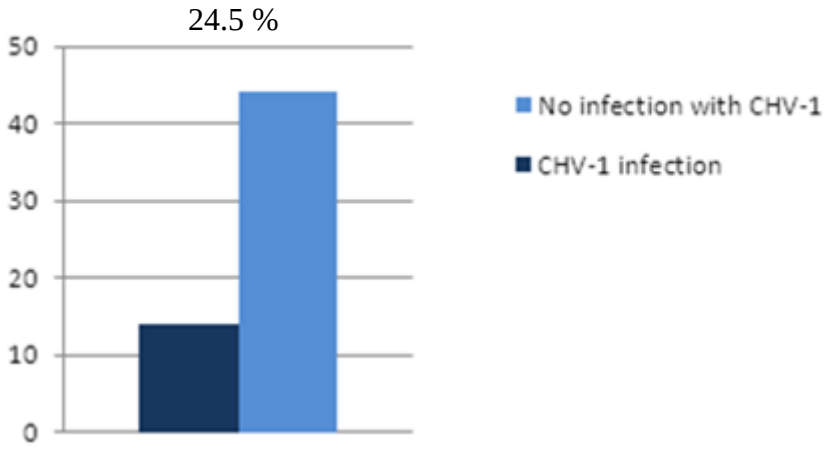

FIGURE 7. Overview of the 14 puppies confirmed positive of CHV-1 by PCR from the population of the submitted puppies. The result represented a prevalence of 24.5%.

4.3 THE PCR RESULTS

The PCR results with corresponding C_t/C_p values of the puppies with CHV-1 infection is given in Table 4.

TABLE 4
Overview of the PCR results from 14 puppies with CHV-1 infection

Puppy no.	Death (Days postpartum)	PCR	Ct/Cp* Liver/Lung	Ct/Cp* Spleen/Kidney	Ct/Cp* Heart
1	6	+	-	-	> 40
2	13	+	13.3	13.8	
3	12 (E)	+	29.5	31.4	
4	2	+	30.6	32.8	
5	3	+	32.9	32.5	
20	12	+	37.8	36.7	
24	0	+	34.0	ND	
27	17 (E)	+	-	>40	
29	0	+	>40	39.4	
30	0	+	34.7	36.9	
49	0	+	35.7	>40	
51	14 (E)	+	38.8	-	
53	1	+	33.6	>40	
56	14	+	31.3	-	

ND: Not done, no tissue avaliable for PCR
(E) : Euthanized
Ct/Cp*: The Ct/Cp values of 1:10 dilution
PCR: + The puppy was positive of CHV-1 infection by PCR

Samples from the liver/lungs and spleen/kidney were collected and pooled in two separate tubes for viral detection of CHV-1 DNA. The 14 puppies listed in Table 4 were positive of CHV-1. The C_t/C_p values were in most of the cases the same for the two pooled samples. However, in puppy no. 49 and 53 (blue squares) the C_t/C_p values of the two pooled samples were different and in puppy no. 27, 51 and 56 (green squares) the virus could only be detected in one of the pooled samples.

In puppy no. 1, the heart had a C_t/C_p value of > 40. No virus could be detected in the liver, lungs, spleen or kidneys, even though these organs have the highest concentration of viral DNA according to the literature (Decaro *et al.* 2010). This puppy was the only one where the heart was analyzed.

The PCR results from the litters with CHV-1 infected puppies and the reported mortality from the litters of the submitted puppy or puppies is given in Table 5.

TABLE 5
The PCR results of the litters with CHV-1 infection and mortality of the litter

Puppy no.	Breed	Death (Days postpartum)	PCR	Ct/Cp Liver/Lung	Ct/Cp Spleen/Kidney	Ct/Cp Heart	Mortality/Littersize
1	Afghan Hound	6	+	-	-	> 40	6/10
2	Boxer	13	+	13.3	13.8		8/9
3	Jack Russell	12 (E)	+	29.5	31.4		1/3
4	Labrador	2	+	30.6	32.8		3/10
5	Labrador	3	+	32.9	32.5		-
6	Labrador	4	-	-	-		-
15	Jack Russell	5	-	-	-		4/5
20	Jack Russell	12	+	37.8	36.7		-
24	Retriever Mix	0	+	34.0	ND		2/2
25	Retriever Mix	0	-	-	-		-
26	Pug	17 (E)	-	-	-		2/2
27	Pug	17 (E)	+	-	>40		-
29	French Bulldog	0	+	>40	39.4		3/8
30	French Bulldog	0	+	34.7	36.9		-
31	French Bulldog	0	-	-	-		-
49	Norwegian Elkhound	0	+	35.7	>40		1/3
51	Bernese Mountain	14 (E)	+	38.8	-		2/11
53	Rottweiler	1	+	33.6	>40		2/8
56	Grand Danoise	14	+	31.3	-		3/13

ND: Not done, no tissue avaliable for PCR
(E) : Euthanized
PCR: + the puppy was positive of CHV-1 infection by PCR, **-**, the puppy was negative of infection
(+): The puppy had low concentrations of viral DNA

Same litters is marked with identical colors

Mortality of a litter with CHV-1 infection is assumed to be high (Decaro *et al.* 2008) as the newborn puppies may acquire the infection from surrounding littermates or from viral secretion of the dam (Greene 2012). Neonatal dogs with CHV-1 infection were detected in 12 different litters (Table 5). From the litters representing the submitted puppies, nine litters had a mortality rate above 80 % (Appendix I). As presented in Table 5 four of the litters with CHV-1 infected puppies had a high mortality rate above 80 %.

An interesting finding was the occurrence of uninfected puppies in litters where CHV-1 infection was present. In five litters not all of the submitted puppies were positive of infection as seen in Table 5. Of the five litters with presence of both infected and uninfected puppies, three of the litters were stillborn puppies.

4.4 PATHOLOGICAL FINDINGS

The pathological findings of the 14 puppies, positive of CHV-1 by PCR are presented in Appendix II.

The gross postmortem findings and occurrences from Appendix II are listed in Table 6.

TABLE 6
Gross postmortem findings and occurrence in 14 puppies positive of CHV-1 infection

Lesions	*N* puppies	Prevalence %
Organomegaly*	7	50 %
Pulmonic edema	6	43 %
*Lesions with hemorrhages***		
In the kidneys	5	36 %
In the lungs	5	36 %
In the liver	4	29 %
In the small intestines	4	29 %
In the spleen	3	21 %
In the subcutis	1	7 %
Congestion (liver, kidney or spleen)	5	36 %
Autolysis of the organs	3	21 %
Congestion and/or hemorrhages of the leptomeninges	2	14 %

* Enlargement of in the liver, kidneys, spleen and/or the thymus
** Hemorrhages characterized as either petechial or ecchymotic

After infection, the virus enters the bloodstream and replicates in vascular endothelial cells lining small blood vessels (Poulet *et al.* 2001; Wright & Cornwell 1968). Of the neonatal dogs with CHV-1 infection, lesions with hemorrhages were presented in about 40 % of the puppies (Table 6). Enlargement of several organs (organomegaly) and pulmonary edema were more consistent findings with prevalence of 50 % and 43 % (Table 6).

Congestion was present in five puppies, which accounted for 36 %. Furthermore, neonatal autolysis had occurred in three of the stillborn puppies at the time of submission which limited the ability to identify characteristic pathological findings (Appendix II).

The association between pathological findings and CHV-1 infection were investigated by statistical analysis in a later section.

4.5 REPRODUCTION AND KENNEL COUGH

Puppies from 37 dams were investigated of CHV-1 infection. The results in the following sections will only include information from 36 bitches, since the owner of puppy no. 23 was unknown.

In accordance to the number of previous litters, 19 bitches did not have any previous litters prior to the study. Of the remaining 17 bitches, nine had one previously litter and the remaining eight had more than one litter as illustrated in Figure 8.

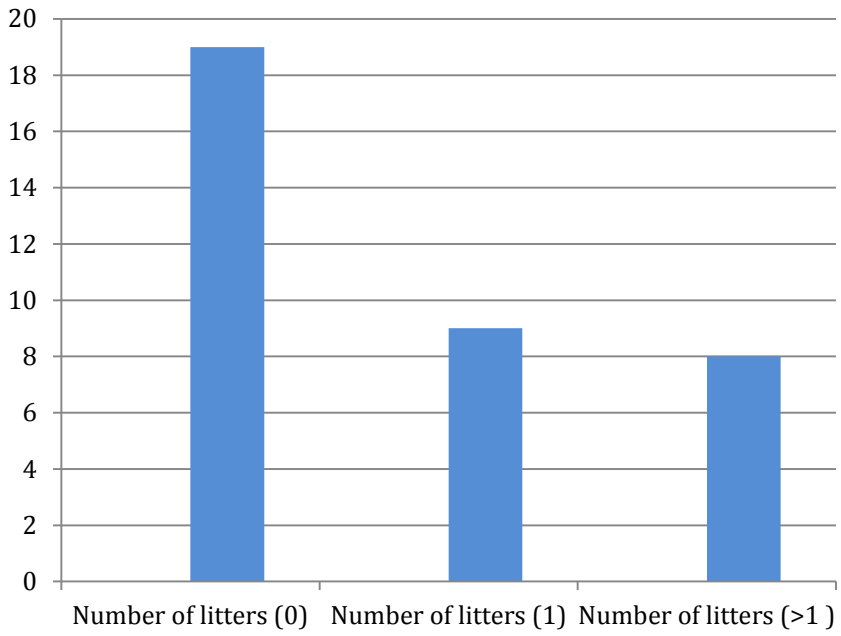

FIGURE 8. Overview of the number of previous litters from 36 bitches. Note that over 50 % of the bitches (n = 19) did not have any previous litters.

The breeders were requested to fill out a questionnaire with general questions about the bitches (Appendix III) particular regarding reproduction disorders. Hashimoto *et al.* (1982) had demonstrated that a virus reactivation or a primary infection of CHV-1 during pregnancy resulted in transplacental transmission to the fetus. Of the 17 bitches with previous litters, 41 % (n = 7) had a history of reproduction disorders as of infertility, stillbirth, signs of abortion or previously litters with high mortality (Figure 9).

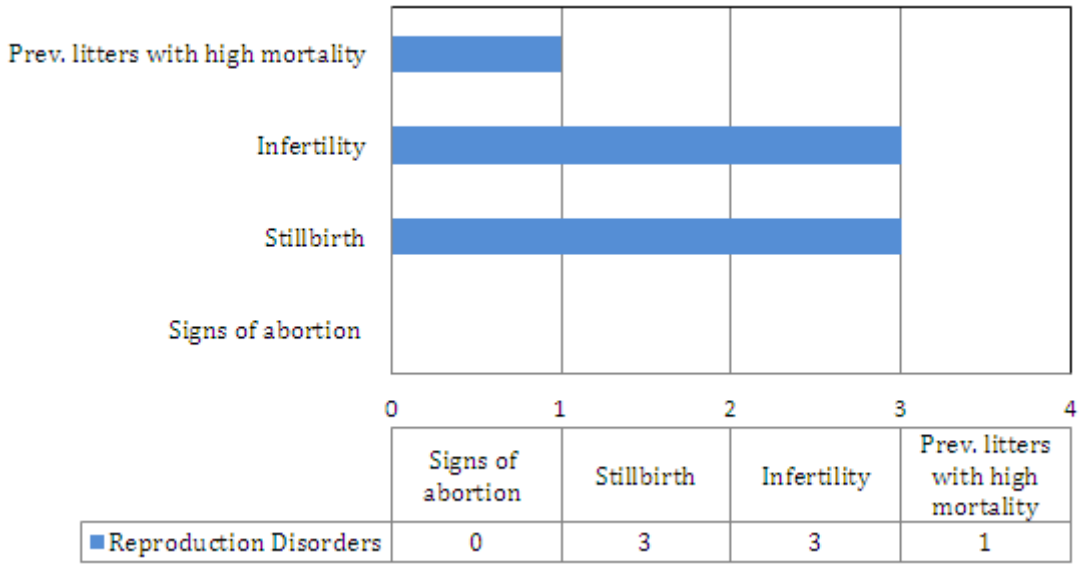

FIGURE 9. Overview of the reproductive disorders from the medical history of seven bitches.

Infertility and stillbirth seemed to be most frequently involved in the reproductive disorders of the bitches. Only one bitch had a previous litter with high mortality. An overview of the litters and dams with a history of reproduction disorders is given in Appendix I.

Of the 36 bitches, 19.4 % (n= 7) had a history of kennel cough. Ronsse *et al.* (2004) demonstrated that dogs with a history of ITB ("Kennel Cough") had a significantly higher antibody titer than dogs without such history. The association between CHV-1 infection and kennel cough are investigated in a later section.

4.6 VACCINATION STATUS OF THE DAMS AND CHV-1 INFECTION

One bitch in the study (Appendix I, see the litter from puppy no. 4, 5 and 6) had been vaccinated with Eurican® Herpes 205 during the pregnancy. The vaccine had been given in a two dose regimen according to the protocol from the manufactures. However, the vaccine had expired two months before use. Otherwise, none of the bitches in the study were vaccinated against CHV-1.

4.7 SIGNIFICANT RISK FACTORS AND PATHOLOGICAL FINDINGS

Risk factors and pathological findings were investigated for association with CHV-1 infection (Table 7 & Table 8).

TABLE 7
Results from logistic regression of risk factors investigated for association with CHV-1 infection.

Risk factors or lesions	*N* dogs	*P**
Age		
1: 0 - ≤ 2 years old	5	0.1487
2: > 2 - ≤ 4 years old	19	0.7135
3: > 4 - ≤ 6 years old	10	0.3417
4: > 6 - ≤ 8 years old	2	0.5275
Kennel size, > 6 dogs	7	0.1815
Kennel cough	7	0.1851
Mortality, > 80 %	9	0.9937
Number of previous litters		
0:	19	0.6340
1:	9	0.4342
> **1**:	8	0.7914
Reproduction disorders	7	0.7785
Vaccinated	1	0.1542
Stillborn	22	0.2268
Clinical signs	11	0.9569
Body condition score (BCS)		
1: Underweight	23	0.3906
2: Normal:	33	0.8488
3: Overweight	2	0.9929

* Values for $P < 0.05$ were considered statistically significant

None of the investigated risk factors were statistical significantly associated with the CHV-1 positive PCR-results and the OR with 95% confidence interval was not calculated.

TABLE 8
Results from logistic regression of pathological findings investigated for association with CHV-1 infection.

Risk factors or lesions	*N* dogs	*P**	*Odds ratio (OR)*	95% Confidence Interval
Lesions with hemorrhages				
In the liver**	4	0.0138	-	-
In the kidneys	5	0.0486	5.5	[0.97 ; 32,2]
In the spleen	3	0.0597	9,6	[0,8 ; 106,6]
In the lungs	5	0.0273	6.4	[1,2 ; 34,5]
In the small intestines	4	0.0229	10.9	[1,3 ; 90,4]
Organomegaly	6	0.0021	13.6	[2,5 ; 74]
Edema in the lungs	7	0.0063	9.3	[1,8 ; 47,3]

* Values for $P < 0.05$ were considered statistically significant
** The *P* value was given through Chi-square test, since none of the PCR negative puppies had lesions in the liver and OR could not be estimated.

The pathological findings with *P* values of < 0.05 are highlighted in red (Table 8). These findings were considered significantly associated with the positive PCR-results. The spleen had a *P* value of 0.0597, which will be considered significant in the following discussion.

The *P* value of lesions in the liver was given through Chi-square test, since none of the PCR negative puppies had lesions with hemorrhages in the liver. Further, OR with corresponding 95% confidence interval could not be estimated for the liver.

	Liver lesions	
PCR	No	Yes
Negative	44	0
Positive	10	4

The presence of organomegaly and pulmonary edema were statistical significant pathological findings associated with PCR-positive puppies, as these lesions were more consistent findings at necropsy (Table 6).

5. DISCUSSION

The pathological findings of CHV-1 infection in dead neonatal dogs and the association between risk factors and pathological findings of CHV-1 infection have been presented in the previous sections. The purpose of these results was to investigate and discuss findings from dead neonatal dogs with CHV-1 infection from the cross-sectional study of 58 puppies and to find the apparent prevalence of CHV-1 infection.

5.1 THE APPARENT PREVALENCE OF CHV-1 INFECTION

The prevalence of CHV-1 infection from the 58 investigated puppies was found to be 24.5 %. The prevalence is considerable higher than other studies of canine neonatal mortality where CHV-1 infection in neonatal dogs and mortality due to the infection seemed to be insignificant.

In a study from Australia of perinatal and late neonatal mortality in the dog, the neonatal mortality from a population of 2574 puppies were investigated. 475 puppies died and five puppies were suspected of infections with CHV-1. Only two puppies which resembles a prevalence of 0.42 % (n = 2/475) were confirmed positive of infection at necropsy and if necessarily through histopathological examination (Gill 2001). Additionally, in a recent study from Norway of neonatal mortality in four large breeds, 127 puppies died with no reports of death caused by CHV-1 infection (Indrebø *et al.* 2007). By email correspondence with Astrid Indrebø (Appendix IV) it was clarified, that the puppies had been examined in the period from 1998-2001 and was part of a larger ongoing research project. At that time, the occurrence of CHV-1 infection in neonatal dogs was supposedly low and the author had no reason to suspect CHV-1 based on the medical history of the puppies. In the litters where the mortality of the puppies was high the puppies were necropsied. Still, there were no characteristically findings of CHV-1 (Indrebø 2013).

In the above mentioned studies the prevalence of CHV-1 infection was either relatively low or not detected. None of the puppies from the two studies was examined by specific viral diagnostic tests of CHV-1 and the diagnosis was based solely on postmortem examination and findings. It can be difficult to find characteristic lesions in the organs from neonatal animals since autolysis may have occurred by the time of submission (Kirkbride 2012). Furthermore, as seen in this thesis in section 5.4, congestion may

further mask hemorrhagic lesions. In these cases, at least histopathological examination of relevant tissue will be necessary in order to identify lesions characteristic for CHV-1 infections, if further detection of viral DNA is not possible. This might explain the discrepancy from the prevalence found in Gill (2001) and Indrebø *et al.* (2007) and the results from this thesis. CHV-1 infection in neonatal dogs might have been underestimated in these studies as there was no special attention or suspicion towards infection at that time. Furthermore, more specific diagnostic methods in detection of CHV-1 were not used.

Even when using more specific diagnostic methods in detection of CHV-1, Rota *et al.* (2007) concluded that CHV-1 was never detected, not even when mortality of a litter was high. The same protocol for PCR diagnostic as Burr *et al.* (1996) established in detection of latent viral CHV-1 with target of the glycoprotein B (gB) gene, was used in the Italian study of neonatal mortality of 66 puppies (Rota *et al.* 2007). The puppies died during the first week of life and were submitted for necropsy in order to find the possible cause of death. In seven litters with suspicion of CHV-1 (due to characteristic postmortem findings at necropsy) pooled samples of tissue from the kidneys, spleen and lungs were analyzed by PCR. None of the puppies from the seven litters was positive of CHV-1. Rota *et al.* (2007) used a different target gene in detection of CHV-1. Otherwise, there is no obvious explanation for the discrepancy between the results reported by Rota *et al.* 2007 and the results from this thesis.

In this study the prevalence of infected puppies was found to be 24.5 % (n = 14). The submitted puppies were randomly selected and evenly distributed throughout the regions of Denmark (Figure 5). The observed prevalence of 24.5% is an estimate of the prevalence of CHV-1 infection on the assumption that the submitted puppies are representative of the entire populations of neonatal dogs that dies during the first three weeks of life. Time constraints limited the number of investigated puppies and due to the lack of earlier studies with similar investigation, it was difficult to quantify a significant sample size representative of the population. In order to validate the prevalence it could be interesting to make a similar study with a larger sample size.

5.2 THE PCR RESULTS AND VIRAL DISTRIBUTION

Of the PCR results (Table 4) the C_t/C_p values were almost the same in the two pooled samples, which would be expected since the virus enters the bloodstream and spreads to several organs causing a generalized infection (Carmichael 1970). In puppy no. 2 the C_t/C_p value was 13.6 (Appendix I) which correspond to a higher amount of viral DNA than the other puppies with values above 30. In puppy no. 27, 51 and 56 the virus could only be detected in one of the pooled samples and in puppy no. 49 and 53 the two concentrations of viral DNA were different between the pooled samples, which may indicate that the virus is not equally distributed in the tissues or that some of the organs have different concentrations of viral DNA. The IDEXX laboratory tested 10 mg of each tissue which may not have contained viral DNA from CHV-1. It would have been interesting to test several pieces of the same tissue in repeated PCR assays to lower the experimental uncertainty.

In puppy no. 1 the heart had a low concentration of viral DNA with a C_t/C_p value of > 40 and no virus could be detected in the liver, lungs, spleen or kidneys. In a study regarding the development and validation of a TaqMan-based real-time PCR, quantification and analysis of CHV-1 DNA in the organs from three naturally infected dogs were performed (Decaro *et al.* 2010). The results revealed that the highest concentration of specific DNA was found in the kidneys, liver, spleen and lungs. In two of the dogs, tissue from the heart was analyzed and even though the virus was present, it had lower concentration of specific DNA than tissues of the kidney, liver, lungs and spleen. Distribution of viral DNA from the results presented in this thesis may be complex and not clearly understood. It might be, that some organs have a natural ability to clear virus from the tissue or that the virus is not proliferating equally in all cell types or parts of the organs, also distribution in the organs may depend on the route of transmission and the time of infection. This could explain the results of unequal distributions of CHV-1 in the organs as discussed above.

An interesting finding in this study was the occurrence of uninfected puppies in litters were CHV-1 infection was present (Table 5). In five litters, not all of the submitted puppies were positive of infection even though the mortality of litters with CHV-1 infection is assumed to be high and may risk mortality rates up to 100% (Decaro *et al.* 2008).

In three litters, CHV-1 infection was confirmed in stillborn puppies. These puppies may have acquired the virus in utero through transplacental transmission. In two of the litters the infection was restricted to only some of the fetuses since not all of the submitted puppies were infected (Table 5, puppy no. 29, 30 & 31). The results resemble the demonstration of transplacental transmission of CHV-1 performed by Hashimoto *et al.* (1983) and Hashimoto *et al.* (1982) with the occurrence of unaffected puppies in litters where the majority of the puppies were infected. When multiple fetuses are present in utero, infectious pathogens may result in different outcomes in different fetuses. The same occurrence has been described in studies of transplacental infection of Aujeszky's disease (Suid Herpesvirus 1). Litters which included both mummified, macerated, stillborn fetuses and live piglets have resulted from inoculation of pregnant sows late in the gestation (Dubovi & Maclachlan 2010).

It is important to emphasize that this study did not aim to investigate the etiology and pathogenesis of CHV-1 in neonatal dogs. Infection in neonatal dogs may be affected by many factors both regarding the immunity of the dams and surrounding dogs as well as the general management and care of newborn puppies. Experimental studies are needed to elucidate and fully understand viral distribution in the organs of infected puppies and occurrences of uninfected puppies in litters were CHV-1 cause infection in some of the puppies.

5.3 CHV-1 INFECTION AND THE CAUSE OF DEATH

The apparent prevalence of CHV-1 infection in neonatal dogs that spontaneously died or was euthanized less than three weeks of age, was found to be 24.5% (n = 14) from the cross-sectional study of 58 puppies. It is important to emphasize that a CHV-1 positive result/infection not necessarily implies that the puppies died from the infection. As described in the literature study, the mortality of the newborn puppies can be related to many other factors. These were not investigated in this study. The virus was confirmed in the puppies by the detection of DNA from the CHV-1 genome and together with characteristic pathological findings of hemorrhages, it was highly indicative of a CHV-1 infection since the virus replicates in the vascular endothelium.

5.4 PATHOLOGICAL FINDINGS

In neonatal dogs the virus enters the bloodstream, resulting in a leukocyte associated viremia with viral replication in vascular endothelium lining small blood vessels, leading to necrotizing vasculitis with secondary hemorrhages (Poulet *et al.* 2001; Wright & Cornwell 1968). Characteristic lesions of CHV-1 infection in neonatal dogs includes petechial to ecchymotic hemorrhages of several organs (Carmichael *et al.* 1965; Wright & Cornwell 1968; Poulet *et al.* 2001). From the necropsy of 58 puppies, eight puppies (no. 2, 20, 27, 46, 50, 51, 55 & 56 in Appendix I) had lesions of hemorrhages in the organs of which CHV-1 tends to replicate and these were suspected of infection. Of the suspected puppies the majority (puppy no. 2, 20, 27, 51 & 56 in Appendix II) were positive of viral detection by PCR. The pathological findings varied in terms of different distribution and prominence of affected organs. Pathological findings of hemorrhagic lesions were highly indicative of a CHV infection, even though other infectious agents can damage the vascular endothelium leading to secondary hemorrhages. Sepsis and endotoxemia is a common cause of endothelial injury (McGavin & Zachary 2007) and Canine Adenovirus (CAV-1) replicates in the vascular endothelial cells and hepatocytes in results of petechial and ecchymotic hemorrhages in the organs of young dogs (Decaro *et al.* 2008). This study was limited to investigate CHV-1 infection in dead neonatal dogs. No other infectious pathogens were investigated and it might be possible that the hemorrhagic lesions were caused by other pathogens or factors with impact on the hemostasis.

Especially lesions of the kidneys are grossly characteristic of CHV-1 (McGavin & Zachary 2007), which is presented with a mottling, heterogeneous appearance of the parenchyma (Poulet *et al.* 2001). Only one puppy (puppy no. 2, Appendix II) had the characteristic lesions in the kidneys. This was also the puppy with the highest concentration of CHV-1 with a C_t/C_p value of 13.6 whereas the remaining puppies had lower viral concentrations with C_t/C_p values above 30 (Appendix I). Findings of hemorrhagic lesions or high concentration of CHV-1 in the organs of puppy no. 2 highly indicated that the puppy died from an infection. However, it would have been necersarry to examine the organs for specific histopathological lesions to confirm a viral replication in the organs, before determining the cause of death.

In addition, it was not always possible to identify the characteristic pathological lesions in neonatal dogs with CHV-1 infection. Hemorrhagic lesions were present in about 40 % of the puppies. This prevalence was expected to be higher, since the virus causes injury to vascular endothelial cells though replication (Poulet *et al.* 2001; Wright & Cornwell 1968). However, it was difficult to identify possible hemorrhagic lesions in the organs of some of the puppies. In puppy no. 24, 29 and 30 (Appendix II) the organs had been subjected to autolysis and further, the spleen and small intestines were missing from puppy no. 24. In puppy no. 3, 4, 5, 49 and 53 (Appendix II) there were signs of congestion in the majority of the organs, which may mask processes producing a light color, such as necrosis (McGavin & Zachary 2007). Congestion may further blur characteristic hemorrhagic lesions of CHV-1. In order to distinguish congestion from hemorrhage, histopathological examination would be necessary.

A more consistent pathological finding was the presence of organomegaly and pulmonary edema. A normal response to cell injury is hydropic degeneration (cell swelling) which results from the failure of the cell to maintain normal homeostasis (McGavin & Zachary 2007). This process can be reversible if the extent and duration of injury is not excessive of affected organs. When cell swelling occurs, the organs will be larger and heavier than normal (McGavin & Zachary 2007). Enlargement of the organs of puppy no. 2, 4, 5, 20, 27, 51 and 56 (Appendix II) may have resulted from acute cell injury of necrosis caused by the virus. Further, pulmonary edema may have resulted from virus replication in vascular endothelium which may have increased the vascular permeability leading to secondary edema. As for the hemorrhagic lesions, it might be possible that organomegaly and pulmonary edema were caused by other pathogens or factors with impact on the cells to maintain normal homeostasis. In order to identify a possible CHV-1 infection, the author believes that pulmonary edema and organomegaly cannot stand alone in order to make a diagnosis. The findings should be seen in conjunction to hemorrhagic lesions as the virus replicates in vascular endothelial cells.

Even as the characteristic pathological findings of the disease were sometimes difficult to identify, the lesions were significantly associated with CHV-1 positive PCR-results.

5.5 SIGNIFICANT RISK FACTORS AND PATHOLOGICAL FINDINGS

Risk factors and pathological findings were investigated for association with CHV-1 infection in dead neonatal dogs. Considerations regarding the risk factors and their possible impact on CHV-1 infection in neonatal dogs will be discussed more detailed in the following section.

Viral shedding from mucosal surfaces during a primary infection in mature dogs is suggested to be prolonged and associated with a higher viral titer than a recurrent infection (Okuda *et al.* 1993; Evermann *et al.* 2011). Seronegative dams which become infected during the first pregnancy could be expected to have a higher risk of transmitting the virus in utero or from viral secretions from mucosal surfaces at postpartum, due to the insufficient levels of antibodies in the serum or colostrum. In order to refine the likelihood of a primary infection during pregnancy, the number of pregnancies in correlation to the age of the bitches was investigated of significant association of CHV-1 infection. The hypothesis was, that bitches in the age from 0 - $\leq$ 2 years old were more likely to have their first litter and could be more vulnerable towards an infection due to a reduced resistance during the first pregnancy as a consequence of the hormonal effects. Though, the number of pregnancies was not statistical significant and neither was the investigation of the age of the bitches (Table 7).

Ronsse *et al.* (2005) showed a tendency of protective antibodies in serum of pregnant dams in the way of reducing the risk of reproductive disorders. Since, reproduction disorders as infertility, abortion and the birth of stillborn puppies may indicate a previously outbreak of CHV-1 (Hashimoto *et al.* 1982; Hashimoto *et al.* 1983) the association between reproduction disorders and CHV-1 infection were investigated for statistical significance. Of the 17 bitches with previous litters, seven bitches had a history of reproduction disorders and the association of reproduction disorders and CHV-1 infection in neonatal dogs was not statistical significant. Some authors suggests that naturally infected dams that give birth to diseased puppies as a consequence of reproduction disorders, will usually give birth to normal litters on subsequent pregnancies (Huxsoll & Hemelt 1970; Evermann *et al.*2011). However, to elucidate the theory in this study a larger number of bitches with a previous history of reproduction disorders would have been needed.

The number of dogs in the kennels and a previous history of kennel cough of the dams investigated by Ronsse *et al.* (2004) showed that these where the primary factors contributing to antibody levels in kennel dogs. None of these factors were statistical significantly associated with CHV-1 infection in this study (Table 7). Neither was the factor regarding the mortality of the litters. From the litters representing the submitted puppies, nine litters had a mortality rate above 80 % (Appendix I) and only four of these litters had puppies with CHV-1. As previously described in the literature study, canine neonatal mortality can be related to many factors that not necessarily involves the presence of CHV-1. Other factors or pathogens which may be involved in the mortality of newborn puppies were not investigated in this study. In summary, none of the investigated risk factors were statistical significantly associated with CHV-1 infection, which may be explained by the limited number of bitches and their disproportional distribution among the defined categories of risk factors (Table 7).

Clinical signs may be presented prior to death of the puppies with CHV-1 infection (Poulet *et al.* 2001). However, clinical signs as a risk factor in this study were not proven to be statistical significant. Neither was the estimation of the body conditions score (BCS). Only the pathological findings were demonstrated to be statistical associated with CHV-1 infections (Table 8). According to the literature, postmortem examination is important in the diagnosis of CHV-1 infection in neonatal dogs (Greene 2012). The results confirm, that the presence of hemorrhagic lesions in the organs of the liver, kidneys, spleen, small intestines and lungs together with the presence of generalized organomegaly and pulmonary edema highly indicates a CHV-1 infection.

In general, only a few dog owners and breeders are interested in founding laboratory test in diagnostics of death puppies. Therefore, CHV-1 infection is likely to be underdiagnosed (Krogenæs *et al.* 2012). If veterinarians would implement routinely postmortem examination in the investigations of pathological causes for mortality in newborn puppies, CHV-1 infections might be confirmed more often in the clinics. The confirmation of an infection would be useful in the surveillance of the virus in order to investigate how often CHV-1 tends to be involved in canine neonatal mortality. Furthermore, breeders and veterinarians would be able to implement prophylactic procedures against CHV-1 in kennels where infections seems to be a problem. An initiative to prevent neonatal losses due to CHV-1 infection could be vaccination of the dams during pregnancy.

5.6 VACCINATION OF THE DAMS

Of the 36 bitches, only one bitch had been vaccinated with Eurican® Herpes 205 during pregnancy. From the litter of the vaccinated dam, three puppies out of ten puppies (n = 3/10) died during the neonatal period (Appendix I). Of the three puppies, puppy no. 4 and 5 were positive of CHV-1 (Table 5). From the medical history of the puppies, the three puppies had nursing problems during the first 24 hours of life and may have received sparse amount of colostrum.

From the pathological findings, puppy no. 4 and 5 (Appendix II) had congestion in the majority of the organs and enlargement of the liver. These pathological findings were not different from puppy no. 6 (data not shown) though, two of the puppies were positive of infection. The pathological findings of puppy no. 4 and 5 were not characteristic of the disease and as discussed in the previous section in order to confirm viral replication and damage in the organs, histopathological examination would be needed. Even though, two of the puppies were positive of infection it does not necessarily imply that they died from this. The results only implied that the puppies were infected.

The fact that only one bitch in this study was vaccinated against CHV-1, confirms the statement that the vaccine is not used or perhaps not recommended in a standard vaccination protocol for pregnant bitches. In the absence of therapy and the poor prognosis of puppies with generalized infection, vaccination of the dams during pregnancy might help veterinarians and breeders to prevent neonatal losses due to CHV-1 infection. However, breeders need to make sure that, when vaccinating the dam during pregnancy the newborn puppies must receive adequate amounts of colostrum during the first few hours of life in order to have the desired effect.

5.7 STASTISTICS

Risk factors and pathological findings associated with infection were analyzed by means of logistic regression analysis to investigate statistical significance. The pathological findings were demonstrated to be significantly association with CHV-1 with *P* values < 0.05 and with OR varying from 5.5 to 13.9. This means that puppies with CHV-1 have 5.5 to 13.9 higher risk of having pathological lesions in the represented organs than uninfected puppies. As specified in the 95% confidence interval of OR (Table 8), the OR values have wide intervals which can be explained by the limited number of puppies in the study. In order to validate the significance of the pathological findings a larger sample size would have been needed, however was not possible due to time constraint.

The pathological findings were not investigated by a multivariable logistic regression analysis since the lesions were depending on each other due to the pathogenesis of CHV-1.

6. CONCLUSION

As to the author's knowledge, there have been no previous investigations focusing on CHV -1 infection associated with canine neonatal mortality. Previous studies have investigated the causes to canine neonatal mortality in general and showed a small prevalence of CHV-1 infections. The infection has therefore not been associated with a high neonatal mortality earlier.

CHV-1 infection in neonatal dogs might have been underestimated in previous studies as there was no special attention or suspicion aimed towards infection at that time and because diagnostics were solely based on postmortem examination and findings. The prevalence of CHV-1 infection from this cross-sectional study of 58 dead neonatal dogs was 24.5% (n = 14) and was considerably higher than expected from previous studies of canine neonatal mortality.

In this study, pathological findings of the infected puppies varied from characteristic petechial and ecchymotic hemorrhages of several organs to non-specific findings, where autolysis and congestion limited the ability to identify relevant pathological lesions. Pulmonary edema and organomegaly were more consistent findings than hemorrhagic lesions during the necropsies. If all of the pathological findings were present in the organs, there was a probability that the neonatal dogs were infected as these findings were demonstrated to have a statistical association with CHV-1 infection with P values < 0.05. If veterinarians implemented routine postmortem examination in the investigations of neonatal mortality, CHV-1 infections could probably be detected in more cases, than the ones/numbers seen today. This would be useful in the surveillance of the virus in order to investigate how often CHV-1 tends to be involved in canine neonatal mortality. A more thorough knowledge would build the foundation for breeders and veterinarians to implement prophylactic procedures against CHV-1 in kennels where infections seems to be a problem.

However, the pathological findings were sometimes difficult to identify and histopathological examination and detection of viral DNA by PCR is needed to confirm diagnosis of CHV-1.

Risk factors and pathological findings were investigated for their association with CHV-1 infection. The pathological findings of hemorrhagic lesions, organomegaly and

pulmonary edema were demonstrated to have a statistical association with CHV-1 infection with *P* values < 0.05, however with a wide 95% confidence interval of OR. In order to further validate the significance of the pathological findings, a larger sample size would have been necessary. None of the investigated risk factors were significantly associated with CHV-1 infection which could be explained by the limited number of bitches and their disproportional distribution among the defined categories of risk factors.

CHV-1 might be an important pathogen in the etiology of canine neonatal mortality. The results from this study demonstrated a relatively high prevalence of CHV-1 infection. However, the infection could not be confirmed as the cause of death. Histopathological examination of the infected puppies could have been valuable in revealing specific lesions. The findings of eosinophilic inclusions bodies would have had a great diagnostic value. By evaluating the extent and duration of the injuries caused by the virus, it would have been possible to determine if the puppies died from the infection. However, findings of hemorrhagic lesions and a high concentration of CHV-1 in the organs of puppies were strongly indicating that the puppies died from the infection. Additional studies on neonatal mortality due to a CHV-1 infection are needed to elucidate this further.

The observed prevalence of CHV-1 infected puppies was 24.5% and is an estimate of the prevalence of CHV-1 infection on the assumption that the submitted puppies are representative of the entire populations of death neonatal dogs. Time constraints and the lack of earlier studies, to quantify a significant representative sample size, limited the number of investigated puppies in this study. In order to validate the prevalence, associated risk factors and pathological findings further studies are necessary with a larger sample size.

The fact that there is no available therapy for puppies with a generalized infection and that this study found a relatively high prevalence of CHV-1, the author suggests that breeders and veterinarians do implement prophylactic procedures including vaccination of the dams during pregnancy in order to protect neonatal dogs from infection.

7. FUTURE PERSPECTIVE

Serological studies have indicated that CHV-1 is a well-known pathogen in the adult dog population in Europe with a prevalence of antibodies against CHV-1 varying from 40-88%. Neutralizing antibodies increases after infection and can remain high and detectable for up to sixty days and it is assumed that that a single serologic test of an animal does not always properly reflect the infection status (Burr *et al.* 1996; Krogenæs *et al.* 2012). Therefore, some authors suggest that the overall seroprevalence in the population might be underestimated. If we consider the entire population of dogs to have a CHV-1 infection either as a latent infection or a virus shedding infection, then why does CHV-1 infection in neonatal dogs occur if we presume that the dams have protective antibodies in the serum and colostrum? Is it due to failure in the uptake of antibodies from the colostrum or is it because we have limited understanding of reactivation and virus shedding from infected dogs?

Ronsse *et al.* 2004 pointed out that a possible explanation might be that other isolates or strains of CHV-1 exists with a different pathogenicity in the airways and the genital tract. The population of healthy dogs may have antibodies against the low pathogenic strain of CHV-1 while a more pathogen strain infects the newborn puppies. Furthermore, some dogs or breeds may be more predisposed to infection and shedding of the virus resulting in transmission of the virus to the newborn puppies. The above-mentioned hypothesis would be very interesting to investigate in future studies.

The results of this study indicate that CHV-1 infection is present in the population of dead neonatal dogs and that the virus might be an important etiological agent in canine neonatal mortality. It would be interesting to investigate the founded prevalence in a more comprehensive study, not only in Denmark but also in other counties, in order to investigate the importance of CHV-1 in the etiology of canine neonatal mortality.

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Appendix I

Raw Data

Overview of the results from the questionnaire, the postmortem examination and the PCR.

For the puppy											For the litter				For the bitch				
Puppy no.	Breed	Gender	Weight (gram)	BCS*	Death (Days postpartum)	Symptoms**	Date of necropsy (2012-2013)	CHV-lesions***	PCR	Ct/Cp**** values at 1:10	Littersize	Stillborn/ littersize	Mortality /litter	Age (years)	Number of Prev. litters	Reproduction disorders	Vaccinated *****	Kennel Cough	Dogs in the household
1	Afghan Hound	F	331	1	6	-	26/9	-	+	**>40**	10	1/10	6/10	6	0	No	No	No	4
2	Boxer	M	795,6	2	13	+	3/10	+	+	**13,6**	9	0/9	8/9	3	2	No	No	No	5
3	Jack Russell	F	160,6	1	12 (E)	-	4/10	-	+	**30,4**	3	0/3	1/3	3	0	No	No	Yes	10
4	Labrador	M	245,3	1	2 (C)	-	16/10	-	+	**32,7**	10	0/10	3/10	7	5	No	Yes	Yes	11
5	Labrador	M	320,1	1	3 (C)	-	18/10	-	+	**32,7**									
6	Labrador	M	368	2	4 (C)	+	18/10	-	-										
7	Terrier Mix	F	114,9	2	0		18/10	-	-		3	1/3	1/3	3	0	No	No	No	0
8	Finnish Lapphund	F	255,8	1	0		31/10	-	-		7	1/7	1/7	6	3	Yes	No	No	4
9	Chihuahua	M	170,3	1	0		1/11	-	-		4	1/4	1/4	4	1	No	No	No	1
10	St. Bernard	M	840	2	0		1/11	-	-		4	3/4	3/4	3	0	No	No	No	3
11	St. Bernard	F	700	2	0		1/11	-	-										
12	St. Bernard	F	660	2	0 (C)		1/11	-	-										
13	Jack Russell	F	152,2	1	0		2/11	-	-		3	2/3	2/3	3	0	No	No	No	1
14	Jack Russell	F	87,4	1	0		2/11	-	ND		-	-							
15	Jack Russell	M	226	2	5	+	2/11	-	-		6	1/6	5/6	2	1	Yes	No	No	0
16	Golden Retriever	M	348,6	2	6	-	2/11	-	-		7	2/7	6/7	6	2	No	No	No	2
17	Golden Retriever	F	273	2	4	-	2/11	-	-		-								
18	Golden Retriever	F	352	2	4	-	2/11	-	-		-								
19	Golden Retriever	F	314	2	4	-	2/11	-	-		-								
20	Jack Russell	M	374	3	12	+	7/11	-/+	+	**37,3**									
21	Dachshund	F	91,6	1	3	-	7/11	-	-		3	1/3	2/3	8	2	No	No	No	0
22	Dachshund	F	61	1	0		7/11	-	-		-								
23							9/11	-	-										
24	Retriever Mix	F	334	2	0		15/11	-	+	**34,02**	2	2/2	2/2	6	0	No	No	No	0
25	Retriever Mix	F	348	2	0		15/11	-	-		-								
26	Pug	F	774	2	17 (E)	+	27/11	-	-		2	0/2	2/2	2	0	No	No	No	2
27	Pug	F	547	2	17 (E)	+	27/11	-/+	+	**>40**	-								
28	Scottish Terrier	F	445	2	10 (E)	+	29/11	-	-		6	2/6	4/6	4	0	No	No	No	3
29	French Bulldog	F	194	2	0		4/12	-	+	**39,7**	8	3/8	3/8	3	1	Yes	No	No	4
30	French Bulldog	F	168	2	0		4/12	-	+	**35,8**									
31	French Bulldog	F	149	2	0		4/12	-	-										
32	Chihuahua	M	84	2	0 (C)		21/12	-	-		4	1/4	2/4	4	1	Yes	No	No	3
33	German Shepherd	M	365,3	2	2	-	21/12	-	-		10	0/10	4/10	4	1	Yes	No	No	1
34	German Shepherd	F	340,6	1	2	-	21/12	-	-										
35	German Shepherd	M	331,6	1	3	-	21/12	-	-										
36	German Shepherd	M	354	1	4	-	21/12	-	-										
37	Basset	F	256	1	4	+	9/1	-	-		8	0/8	3/8	5	0	No	No	Yes	25
38	Beagle	M	175	1	5	+	10/1	-	-		7	0/7	2/7	6	3	No	No	No	3
39	Beagle	M	192	1	6	+	11/1	-	-										

For the puppy											For the litter			For the bitch						
Puppy no.	Breed	Gender	Weight (gram)	BCS*	Death (Days postpartum)	Symptoms**	Date of necropsy (2012-2013)	CHV-lesions***	PCR	Ct/Cp**** values at 1:10	Littersize	Stillborn/ littersize	Mortality /litter	Age (years)	Number of Prev. litters	Reproduction disorders	Vaccinated *****	Kennel Cough	Dogs in the household
40	Danish Mastiff	F	380	2	1	+	6/2	-	-		12	1/12	2/12	4	0	No	No	No	2
41	Coton De Tulear	M	207	1	8 (E)	-	7/2	-	-		4	0/4	2/4	4	1	No	No	Yes	1
42	Shih Tzu	F	286	2	5	-	8/2	-	-		6	0/6	1/6	2	0	No	No	Yes	5
43	Chihuahua	M	80	1	10	-	13/2	-	-		5	1/5	4/5	5	5	Yes	No	No	9
44	Chihuahua	M	87	1	4	-	13/2	-	-		5	0/5	1/5	2	0	No	No	No	9
45	French Bulldog	F	80	1	0		13/2	-	-		6	1/6	2/6	6	0	No	No	No	2
46	Jack Russell	F	255	2	0 (C)		16/2	-/+	-		3	3/3	3/3	6	0	No	No	No	0
47	Jack Russell	F	204	2	0		16/2	-	-										
48	Jack Russell	F	190	1	0		16/2	-	-										
49	Norwegian Elkhound	M	130	2	0		19/2	-	+	**37,8**	3	1/3	1/3	4	0	No	No	No	1
50	Boxer	F	780	2	6 (C)	-	19/2	-/+	-		6	0/6	1/6	3	1	No	No	No	3
51	Bernese Mountain	F	913	3	14 (E)	+	22/2	-/+	+	**38,8**	11	1/11	2/11	4	0	No	No	No	15
52	Newfoundland	M	485	2	0 (C)		22/2	-	-		10	1/10	1/10	4	1	Yes	No	Yes	10
53	Rottweiler	M	340	2	1	-	28/2	-	+	**36,8**	8	0/8	2/8	2	0	No	No	Yes	1
54	Rottweiler	F	260	2	13	+	1/3	-	-		7	3/7	7/7	5	0	No	No	No	0
55	Boxer/Labrador Mix	F	480	2	0 (C)		9/3	-/+	-		1	1/1	1/1	3	0	No	No	No	0
56	Grand Danoise	M	1480	2	14	-	13/3	-/+	+	**31,3**	13	0/13	3/13	5	10	No	No	No	0
57	German Shepherd	F	330	1	2	+	21/3	-	-		8	1/8	5/8	4	1	No	No	No	3
58	German Shepherd	M	450	2	2	+	21/3	-	-										
													n = 99/224						

* BCS in groups of: 1 (Underweight) 2: (Normal) 3: (Overweight)

** Symptoms: **(+)** if ≥ two different symptoms **(-)** if < two different symptoms

***CHV-1 lesions: **(+)** if characteristic gross pathological lesions of CHV-1 was present,

(-/+) if present of hemorrhagic lesions in several organs and suspect of CHV-1 infection **(-)** if no characteristic lesions

****Ct/Cp values at dilution 1:10. This value represents the mean value of liver/lung and spleen/kidney.

Specific Ct/Cp values are summarized in Table 4.

***** Vaccinated with Eurican® Herpes 205 during the pregnancy

Appendix II

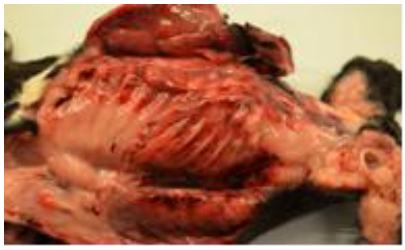
Pathological findings in CHV-1 infected puppies

All of the photos are available on the attached CD-ROM

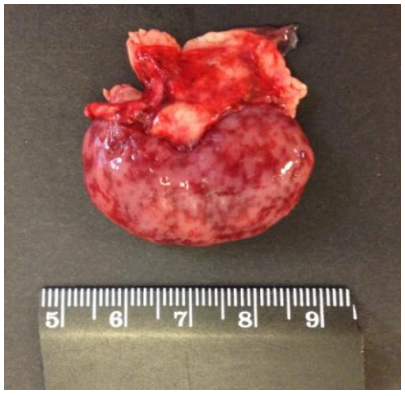
Puppy no. 1

Gross pathology: Diffuse subcutaneous hemorrhages on the thorax and abdomen (see pictures). Bilateral petechial hemorrhages in the kidneys. Ten ml of unclear fluid with fibrin clots in the thorax.

Age: 6 days.

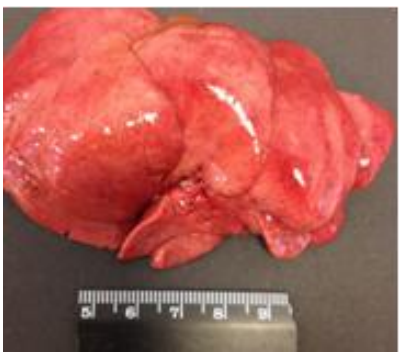


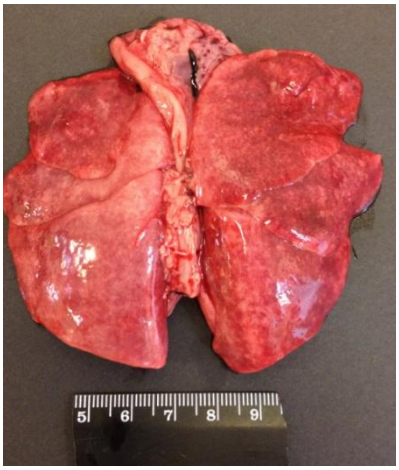
Puppy no. 2

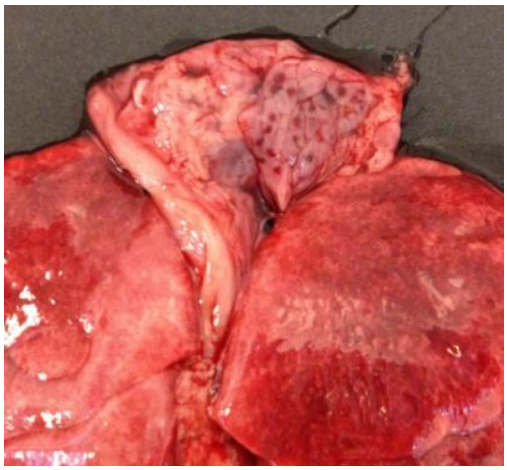


Gross pathology: Petechial to ecchymotic hemorrhages in the thymus, liver, lung and the kidneys (see pictures). There organs were enlarged especially the spleen, liver and kidneys (organomegaly). In the small intestines there were hemorrhages in the area of the Peyer's patches of the ileum. Small amount of serohemorrahagic fluid in the thorax. The lungs had edema and failed to collapse.

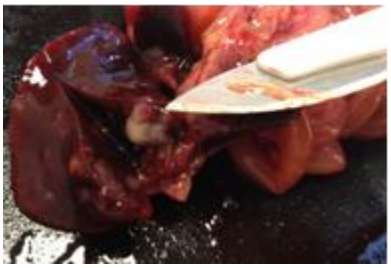
Age: 13 days.







Puppy no. 3



Gross pathology: Congestion in the liver and kidneys. In the liver, area of lobus dexter pars lateralis with multifocal small processes of mucopurulent content. Pulmonary atelectasis/consolidation in the cranioventral lobes of the lung. The lungs failed to collapse in the dorsocaudal region due to edema.

Age: 12 days. Euthanized by the veterinarian with pentobarbital intraperitoneally.

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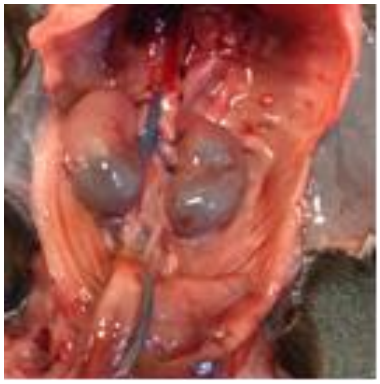
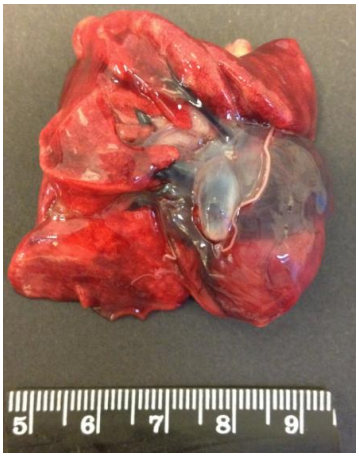
Puppy no. 4



Gross pathology: A small area around the umbilicus with hemorrhages. Congestion and enlargement of the liver and kidneys. In the right kidney, congestion was only present in the caudal pole. In both kidneys, congestion was localized to the medulla.

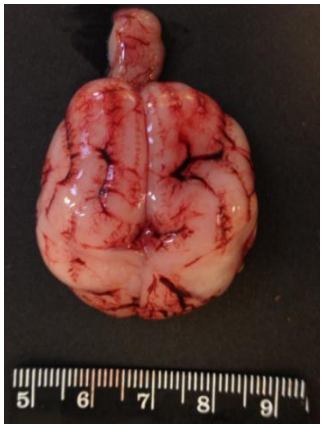
Puppy no. 4

A cyst with serous contents was found above the heart (see picture) and petechial hemorrhages was present in vena cava caudalis.



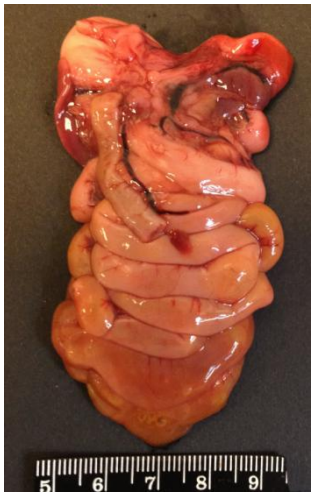
Age: 2 days.

Puppy no. 5

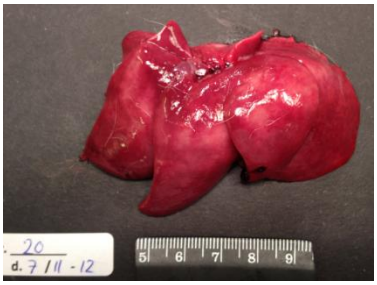


Gross pathology: Renal congestion similar to puppy no. 4 (Same litter). Hepatic congestion and hepatomegaly. Edema in the small intestine and autolysis. Pulmonary edema. Mild meningeal congestion.

Age: 3 days.

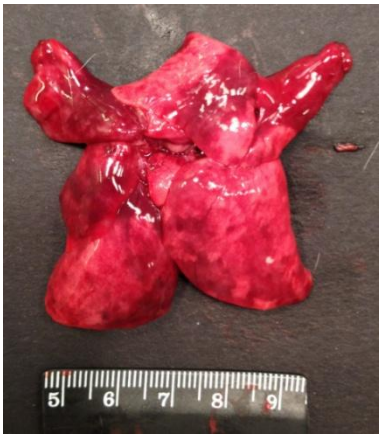


Puppy no. 20

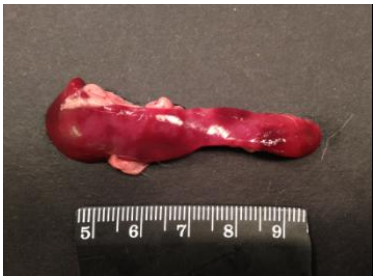


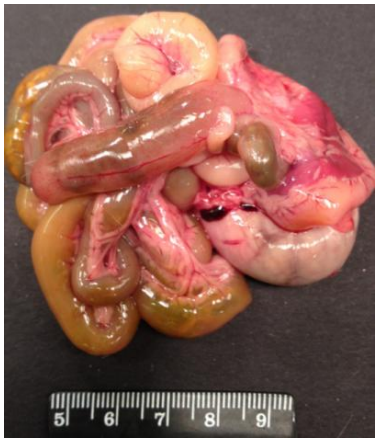
Gross pathology: Serous fluid in the abdominal cavity. Hepatomegaly and hepatic and splenic ecchymoses. Liver seemed fragile. Edema in the small intestine and petechial hemorrhages, especially in the ileum. Renal petechial hemorrhages.

Pulmonary edema and lungs failed to collapse. Heterogeneous and mottling appearance of the lung with areas of red and white patches.

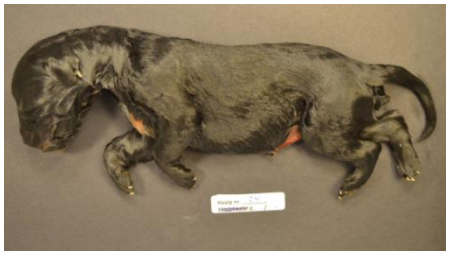


Age: 12 days.





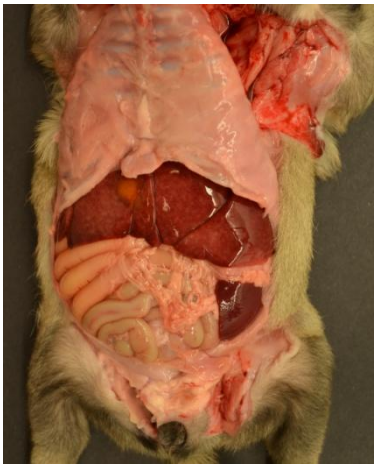
Puppy no. 24

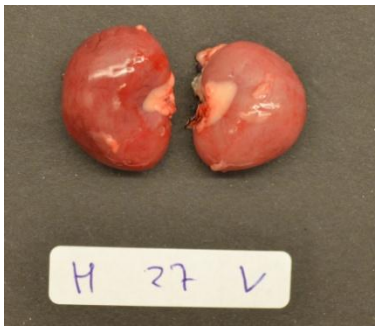


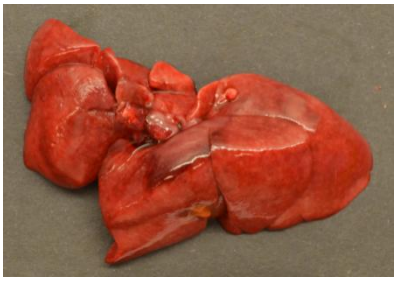
Gross pathology: Autolysis and serohemorrhagic fluid in the thorax. Diffuse congenital pulmonary atelectasis. The spleen and small intestines were missing, probably eaten by the dam.

Age: Stillborn.

Puppy no. 27







Gross pathology: Hepatomegaly, splenomegaly and renomegaly. Disseminated pinpoint necrosis in the liver. Focal area of hemorrhages in the right kidney. Lungs were not fully aerated (pneumonia?) The lateral ventricles appeared enlarged (hydrocephalus?).

Age: 17 days. Euthanized by the veterinarian with pentobarbital intracardially.

Puppy no. 29

(No pictures)

Gross pathology: Autolysis. Diffuse congenital pulmonary atelectasis.

Age: Stillborn.

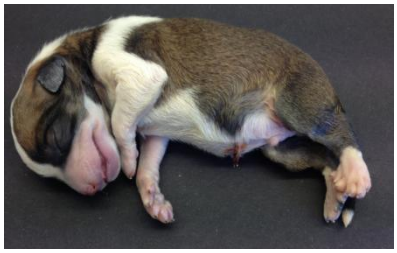
Puppy no. 30

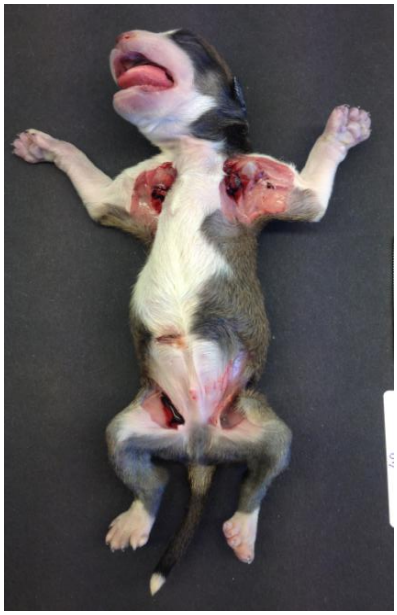
(No pictures)

Gross pathology: Autolysis. Diffuse congenital pulmonary atelectasis.

Age: Stillborn.

Puppy no. 49





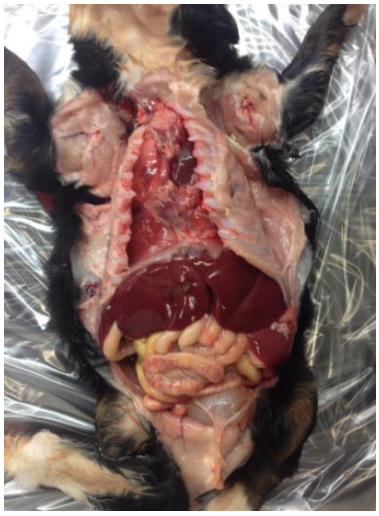
Gross pathology: Slight bloody exudate from the nares. Congestion in the liver and kidneys. The small intestine was dark red and congested.

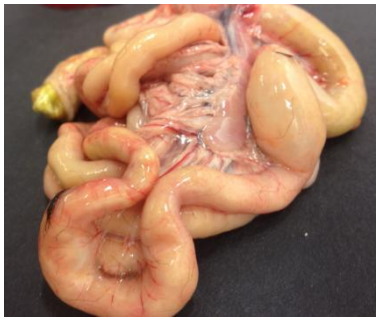
Diffuse congenital pulmonary atelectasis.

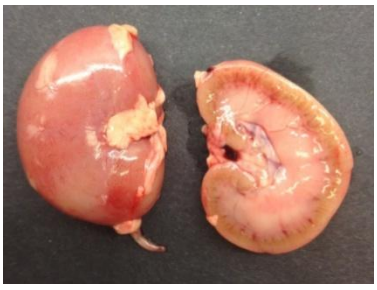
Mild leptomeningeal congestion.

Age: Stillborn.

Puppy no. 51



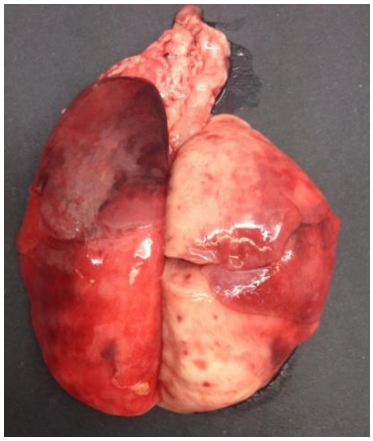


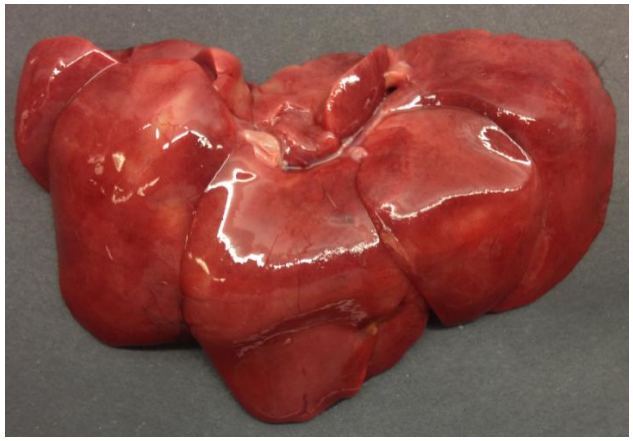


Gross pathology: Distended urinary bladder with white to yellow sediments. Spleno-, reno – and hepatomegaly. Slight enlargement of the intestinal/mesenterial lymph nodes. Petechial hemorrhages in the ileum and ecchymotic hemorrhages in the kidneys.

Edema in the left side of the lung. The right half of the lungs were pale and with presence of mineralization due to pentobarbital.

Age: 14 days. Euthanized by the veterinarian with pentobarbital in the thorax.



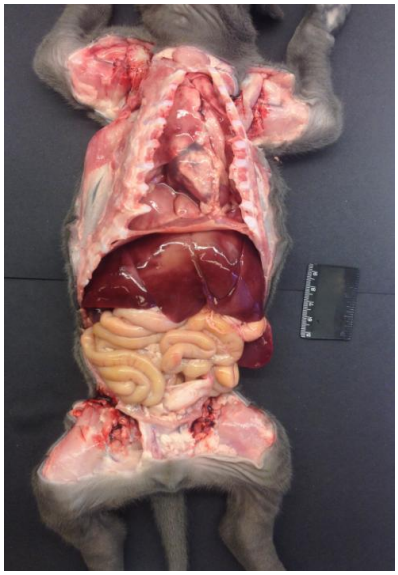


Puppy no. 53

Gross pathology: Congestion in the liver and possible petechial hemorrhages in the spleen (not clearly visible). Milk in the stomach. Hemorrhages on the top of the skull, especially around the eyes.

Age: 1 day.

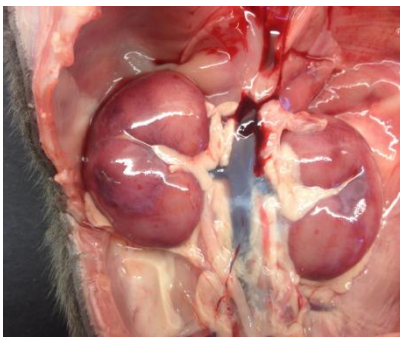
Puppy no. 56

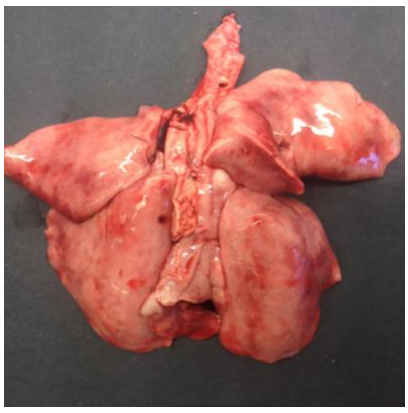


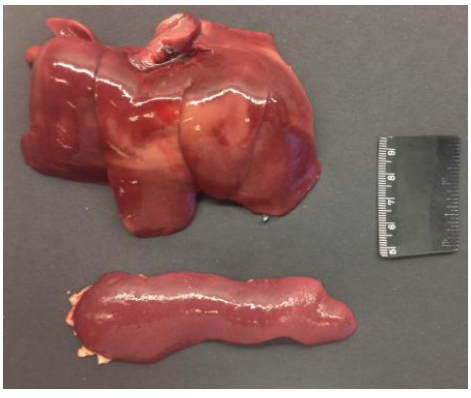
Gross pathology: Multifocal purulent folliculitis in the ventral part of the neck. Yellow feces in the rectum.

The small intestine was pale and with petechial hemorrhages in the ileum. The liver and spleen were enlarged (see picture). The kidneys were enlarged and with ecchymotic hemorrhages (see picture). The lungs failed to collapse with presence of edema and had a heterogeneous appearance.

Age: 14 days.







Appendix III

Herpesvirusundersøgelse

Dato for indlevering af hvalp/hvalpe: _____ Antal hvalpe: _____

Ejer:

_____

Adresse:

_____

Postnr/by:

_____

Tlf:

_____

E-mail:

_____

Klinik (stempel)

Spørgsmål for tæven

Tævens navn: _____

Race: _____

Dato for kuldfødsel: _____

Antal levende hvalpe: _____ Antal dødfødte hvalpe: _____

Har tæven før været drægtig? Ja / Nej
Hvis ja, antal kuld: _____

Hvis ja, har tæven før haft reproduktionsproblemer?:
Gået tom / Aborteret / Født dødfødte hvalpe

Er tæven blevet herpesvaccineret? _____ Hvis ja, hvornår? _____

Har tæven før haft symptomer/blevet diagnosticeret med Kennelhoste?
Ja / Nej

Er der andre hunde i husstanden foruden tæven og hendes hvalpe?
Ja / Nej Hvis ja, hvor mange? _____

Spørgsmål for hvalpen

Hvilken status havde hvalpen ved fødslen: Levende / Dødfødt

Hvis levende, hvor mange dage efter fødslen døde hvalpen: _____

Hvis levende, blev hvalpen aflivet hos dyrlægen: Ja / Nej

Hvis levende, var hvalpen tilsyneladende rask ved fødsel: Ja / Nej

Hvis levende, har hvalpen udvist symptomer såsom (afkryds):

Flåd fra næsen / Nysen / Ukoordinerede bevægelser
Diarre / Appetitløshed / Volkalisering / Kulderystelser/
Ingen symptomer

Er hvalpen blevet **behandlet hos dyrlægen**? Ja/Nej

Hvis ja, hvilken behandling har hvalpen fået? _____

Har hvalpen været i kontakt med andre hunde end tæven og kuldsøskende? Ja/Nej

Ved indlevering af mere end én hvalp bedes spørgsmålene (se ovenfor) besvares ud fra hver enkelt hvalp

Ejer skal være inforstået med at hvalpen obduceres og undersøges for dødsårsag, især forårsaget af CHV-1. Dette gøres ud fra en obduktion, samt påvisning af virus ved mikroskopering og PCR af udvalgte væv/hvalpe.

Ved slutningen af undersøgelsen vil alle hvalpe som indgår i dette studie blive sendt til fælleskremering via Universitetshospitalet for Familiedyr

Den specialestuderende (Rikke Wendt Larsen) vil i slutningen af undersøgelsen, ud fra ovenstående personlige kontaktoplysninger om ejeren kontakte ejer med resultatet af laboratorieprøverne,
Ejer kan forvente svar i foråret 2013

Ejer er med denne signatur inforstået med ovenstående

Underskrift(ejer):_____

Appendix IV

Email correspondence with Astrid Indrebø

Veterinarian, dr. scient with PhD in obstetrics. Head of Nordic Kennel Club, Norwegian Kennel Club.

From the Astrid Indrebø:

Kjære Rikke!

Det ble ikke foretatt regelmessige obduksjoner I vårt materiale. Dette var det helt opp til oppdretter selv å avgjøre, men vi anbefalte at døde valper skulle obduseres. Prosjektets hovedmål var imidlertid å studere utvikling av skjelettlidelser, og for dette formål var de levende valpene naturlig nok av størst interesse.
Valpene som inngår i denne undersøkelsen var født i tidsrommet 1998-2001. På det tidspunktet var forekomsten av CHV-1 i Norge antatt å være svært lav. På bakgrunn av de opplysningene som er gitt av oppdretter er det liten grunn til å tro at CHV-1 hadde noen betydning for at valper døde. I enkelte kull med stor valpedødlighet ble valper obdusert, men det ble ikke gjort obduksjonsfunn forenelige med CHV-1.

Jeg gleder meg virkelig til å lese din artikkel. Det er svært interessant tema du arbeider med!

Mvh
Astrid Indrebø