An investigation of Kennel cough in Sweden focusing on canine calicivirus

Zeeshan Afzal

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Zeeshan Afzal

Supervisor: **Maja Malmberg, Swedish University of Agricultural Sciences, Department of Biomedical Sciences and Veterinary Public Health**

Assistant supervisor: Jonas Johansson Wensman, Swedish University of Agricultural Sciences, Department of Clinical Sciences

Examiner: Mikael Berg, Swedish University of Agricultural Sciences, Department of Biomedical Sciences and Veterinary Public health

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Abstract

Kennel cough, which is also known as the Canine Infectious Respiratory Disease (CIRD) is a disease complex caused by several different pathogens including both viruses and bacteria. According to previous research, canine adenovirus, canine parainfluenza virus and *Bordetella bronchiseptica* are common causes of kennel cough. Recently, other pathogens found to cause or be associated with kennel cough are canine respiratory coronavirus and *Mycoplasma* species.

Research at the Swedish University of Agricultural Sciences is ongoing to explore novel pathogens that may play a role in kennel cough, and one such candidate pathogen is canine calicivirus. Before starting this project, an initial metagenomics study was performed on samples from 14 dogs of which 12 were positive for CIRD and 2 from healthy individuals. According to the metagenomic study 2 out of the 12 dogs with CIRD were found positive for canine calicivirus, one of which was only positive for canine calicivirus and no other pathogens. So, the question arises if canine calicivirus has any role in CIRD? To answer this question 75 samples from dogs with (n=59) and without (n=16) clinical signs of kennel cough were screened for canine calicivirus using a specific calicivirus RT-PCR. The result shows (7 out of 59) (11.86%) dogs with clinical signs of kennel cough were tested positive for canine calicivirus, and no dogs (0 out of 16) tested positive for canine calicivirus from the healthy control group.

The 07 dogs that were tested positive for canine calicivirus in the present study except for one dog displayed mild signs. Overall, our results indicate that there could be an association between canine calicivirus and kennel cough, something that has previously not been described. To date, this is also to our knowledge the first description of canine calicivirus in dogs in Sweden.

Keywords: Kennel cough, Canine Infectious Respiratory Disease, Canine Calicivirus, Canine parainfluenza virus, *B. bronchiseptica*, Sweden

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Abbreviations

CIRD	Canine Infectious Respiratory Disease
CPIV-2	Canine parainfluenza virus type 2
PCR	Polymerase chain reaction
RT-PCR	Reverse-Transcriptase- polymerase chain reaction
CAV-2	Canine adenovirus type 2
RNA	Ribonucleic acid
DNA	Deoxyribonucleic Acid
cDNA	Complementary DNA
CaCV	Canine Calicivirus
CRCoV	Canine Respiratory Coronavirus
SVA	National Veterinary Institute
SLU	Swedish University of Agricultural Science
FCV	Feline Calicivirus
VESV	Vesicular exanthema of Swine Virus
ICTV	International Committee on Taxonomy of Viruses
CPE	Cytopathic effects
WRAIR	Walter Reed Army Institute of Research
CaVV	Canine *vesivirus*
CIV	Canine influenza virus
Bb	*Bordetella bronchiseptica*

1 Introduction

Kennel cough, also called Canine Infectious Respiratory Disease (CIRD) or infectious tracheobronchitis, is a contagious respiratory disease complex affecting dogs. It commonly involves the upper respiratory tract and is caused by several infectious agents in combination or alone (Appel & Binn 1987; Mochizuki et al. 2008). Pathogens commonly associated with CIRD include canine parainfluenza virus type-2 (CPIV-2), canine adenovirus type-2 (CAV-2), and *Bordetella bronchiseptica* (Bb). However, several other pathogens have also been identified as being part of the complex, including *Mycoplasma cynos*, canine respiratory coronavirus (CRCoV), *Streptococcus* spp., and canine Pneumovirus (Mitchell et al. 2013; Erles et al. 2004; Chalker et al. 2004). *B. bronchiseptica* (Bb) is commonly isolated from infected animals and experimental infection using this pathogen produces typical clinical signs of kennel cough (Erles et al. 2004). Canine parainfluenza virus type-2 (CPIV-2) is also commonly isolated from infected animals, and it has also been demonstrated to cause mild infections (Ford 2012).

Canine respiratory coronavirus (CRCoV) was first identified in a rehoming center UK that had a high incidence of canine infectious respiratory diseases (Erles et al. 2003). In different countries, canine respiratory coronavirus (CRCoV) is highly prevalent among dogs that show clinical signs of respiratory disease (Mochizuki et al. 2008). For example, Schulz et al. (2014) found CRCoV to be present in 9.8% of dogs in Germany with CIRD as compared to healthy dogs where CRCoV was shown to be absent. This result indicated that CRCoV is present in the German dog population. Additional surveys show antibodies against CRCoV have been detected in dogs in different countries worldwide including Italy (Prestnall et al. 2007), New Zealand (Knesl et al. 2009), UK and Ireland (Prestnall et al. 2006). A recent survey was performed by Wille et al. (2020) in Sweden to investigate the prevalence of CRCoV in Swedish privately owned dogs. Samples were collected in years (2013-2015) from different locations in Sweden. Wille et al. (2020) have found CRCoV in 13 dogs (14.7%) after the screening of 88 dogs that were suffering from canine Infectious respiratory disease (CIRD). Moreover, as healthy control, 20 dogs were

used and none of the dogs were tested positive for CRCoV from healthy control (Wille et al. 2020). In 2004, the canine influenza virus causing severe respiratory disease was detected in a racing greyhound in the USA (Crawford et al. 2005).

Worldwide, dogs are generally vaccinated against the principal pathogens of CIRD, *B. bronchiseptica* and CPIV-2, but outbreaks of CIRD continue to be reported throughout the world. The vaccines are unlikely to provide complete immunity against all the different causative agents, especially because to date, there are no vaccines available for some of the pathogens such as CRCoV, *Mycoplasma species* and Pneumovirus for dogs. Furthermore, there are also some unknown causative agents of CIRD (Erles et al. 2003). As the disease complex is generally diagnosed based on the characteristic coughing, there is no routine practice of laboratory diagnosis to further investigate the causative agents. Therefore, limited information is available for the aetiology of CIRD. Thus, there is a need to determine novel causative agents of CIRD, which would help to design better vaccines and diagnostic tools in order to control CIRD. A research investigation of kennel cough in Sweden is ongoing at the Swedish University of Agricultural Sciences (SLU). From 2013-2016, dogs with CIRD and healthy dogs were enrolled and screened for the main causative agents of CIRD.

1.1 Aim

An initial viral metagenomics study was performed on 14 of these dogs (12 with CIRD and 2 healthy). Sequence data suggested presence of a tentative canine calicivirus in 2 out of the 12 dogs with CIRD. One of these dogs was not positive for any other known agents, raising the question: Is canine calicivirus a possible cause of CIRD in dogs? In this thesis, the primary focus is to address this question by assessing the prevalence of canine calicivirus among Swedish dogs and investigating a possible association with CIRD.

2 Literature review

2.1 Clinical signs

Kennel cough is a disease complex with many bacteria and viruses inducing clinical signs. CIRD is known to be the most prevalent infectious disease of dogs (Ford 2012). In this disease, the symptoms can vary from being mild signs to being severe. The duration between exposure to the development of symptoms ranges from 3 to 10 days, regardless of the causative agents involved in the primary infection. In some cases, a secondary bacterial infection may occur which may cause a more general condition. The secondary infection can also cause pneumonia (Ford 2012). In physical examinations, dogs suffering from CIRD often have a short-term fever, and maybe lethargic, loss of appetite and laboured breathing. (Ford 2012).

2.2 Epidemiology

The mode of transmission of the pathogens causing CIRD is mostly through direct contact with sick dogs or by airborne contact through infectious respiratory secretions (Buonavogia & Martella 2007). *B. bronchiseptica* can replicate and survive at 37 °C for at least 21 days (Porter et al. 1991). *Mycoplasma* species and *B. bronchiseptica* shedding can occur for three months or more (Appel & Binn 1987). Usually, the first cough develops in infected dogs within 3 days after exposure. The host ranges include cats, rodents and wildlife (Ford 2012). Most outbreaks occur due to direct contact of dog-to-dog or due to airborne contact with respiratory secretion, while some studies have suggested that pathogen, *B. bronchiseptica* can also spread from dogs to cats (Ford 2012).

When CIRD is caused by a virus, the virus shedding starts a few days after the infection (Ford, 2012). The duration of the Canine parainfluenza virus shedding is 6 to 10 days and then the load of the virus reduces significantly (Ford 2012).

However, common causative agents of CIRD are spread up to 14 days after infection. A greater risk of infection has been seen among animals that are kept in kennels, veterinary hospitals and pet shops compared to animals that are kept indoors (Appel & Binn 1987; Bemis DA. 1992).

2.3 Diagnosis

Clinical diagnosis is based on the history of recent exposure to dogs, symptoms displayed and observed response to empiric therapy. Moreover, in some cases, information about the recent history of vaccination can also be helpful for diagnosis. However, a history of recent vaccination (within 6 months) in a dog with characteristic respiratory signs does not exclude a diagnosis of kennel cough. For practicing veterinarians, the use of molecular techniques for the detection of DNA or RNA from infectious agents is increasing in the commercial setting (Ford 2012). Fluid taken from transtracheal aspirates may provide information on the neutrophilic exudate. However, to confirm bacterial pneumonia and prescribing appropriate therapy bacterial culture of aspirated fluid may be helpful (Hawkins 1995 see Ford 2006).

Bacterial isolates that were taken from swabs of the nasal and oral cavities, nasopharynx and oropharynx do not necessarily show primary or secondary pathogens due to indigenous microflora. Although, bacterial samples that were obtained from transtracheal aspiration fluid, endotracheal or bronchoalveolar lavage have more chances to show causative agents that cause the disease. Studies by Hallender (1999) also revealed that nasopharyngeal swab collection yields fewer bacteria comparative to aspiration of lavage fluids because these bacteria adhere to fiber-tipped swabs that were made of cotton, alginate, or Dacron.

Differential diagnosis

CIRD can be confused with other diseases like airway foreign body, cardiogenic and non-cardiogenic pulmonary edema, and pulmonary hemorrhage (Cohn & Langdon 2008).

2.4 Treatment and management

Kennel cough is, in most cases, a transient disease, and most dogs that have the infection are cured without any medical treatment. The dog may need veterinary treatment in more severe cases when the general condition of the dog is affected and deep coughing. One study shows that the administration of an oral or parenteral antibacterial agent can reduce the duration of coughing in infected animals

(Thrusfied 1991). The drugs that had good results were trimethoprim- sulfonamide and amoxicillin. These drugs are effective because the dogs with clinical signs of CIRD may be at an increased risk of bacterial bronchopneumonia, thus administration of empirical antimicrobial drugs could be justified (Vaden & Papich 1995 see Ford 2006). Doxycycline is preferred for its efficacy against *B. bronchiseptica*, it can be given orally (tablets, liquid suspension, capsules) or it can be administered a single dose daily to less than 10 days to minimize the risk of infection (Ford 2012). If lower respiratory tract infection such as (bronchopneumonia or interstitial pneumonia or sepsis) is suspected, then intravenous antimicrobial treatment is indicated. Ideally, the antimicrobial treatment should be based on susceptibility results and results of bacterial culture; however, empiric antimicrobial treatment is considered most appropriate in the clinical setting (Ford 2012).

A previous study has shown that *B. bronchiseptica* isolated from cats in Liverpool and California are resistant to many antibiotics including sulfonamides, fluroquinolones, and some cephalosporins (Foley et al. 2002). Furthermore, another study by Ellis et al. (2001) indicated that *Bordetella* vaccine both intranasal and parenteral gives similar protection after exposure to the organism based on clinical signs, antibody titers, upper airway culture, and histopathologic examination. Although, vaccines against *B. bronchiseptica* and CPIV-2 are available in the market. Even regular vaccinations have failed to provide sterile immunity or complete protection against the infection (Day et al. 2016).

Particularly in a high-intensity population, the vaccine may not guarantee protection against this disease (Ford 2012). The complete routine of the cleaning is being suggested to be necessary. This includes adequate ventilation from 12 to 20 air exchange per hour in the house where dogs are kept (Ford 2012). Isolation of the dogs is necessary to further reduce the spread of the disease (Ford 2012). Moreover, studies have also demonstrated that stress can have negative impact on the immune response to a pathogen, so methods that help to reduce the stress and barking may be helpful to control the disease (Millor 2004).

Prognosis

CIRD prognosis is excellent in uncomplicated infection. In secondary bacterial infection, the dog gets a fever, impaired general condition, and nasal flow, and in serious cases, it may cause fatal consequences. This is primarily a risk for puppies and immunosuppressed individuals (Ford 2012).

2.5 Pathogenesis and main Causative agents

Despite available vaccines, CPIV-2 and *B. bronchiseptica* are the most common pathogens in the outbreak of kennel cough. (Decaro et al. 2016; Englund et al. 2003; Erles et al. 2004). This is because the vaccines available CPIV-2 and *B. bronchiseptica* do not give adequate protection to prevent disease (Day et al. 2016). However, it was observed in many cases of CIRD that *B. bronchiseptica* is not present in the respiratory tract (Ueland 1990) and serological data does not support *B. bronchiseptica* presence (Chakler et al. 2003 a,b). In the text below, the various pathogens, their characteristics, and pathogenesis are described.

2.6 Canine Parainfluenza Virus

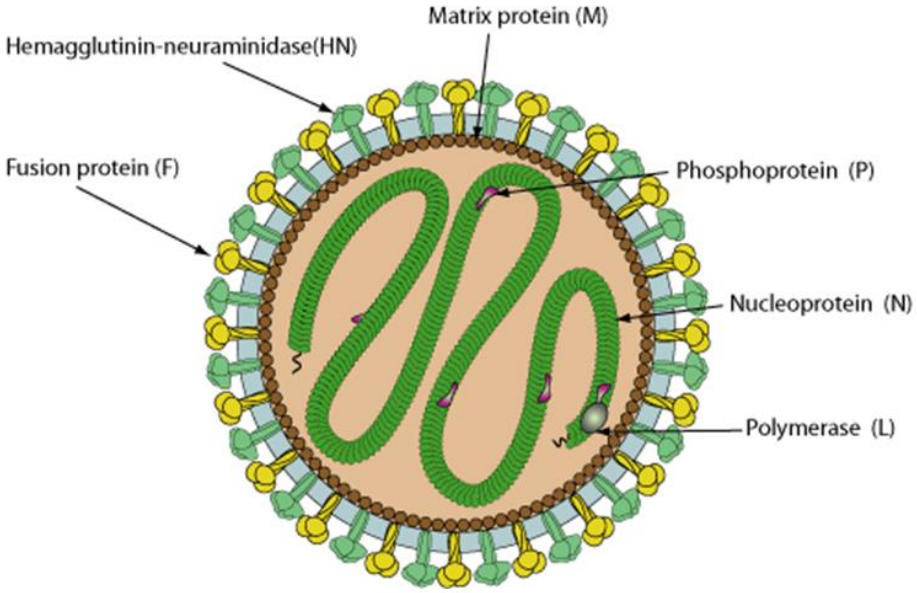

Figure 1. Illustrated from Swiss Institute of Bioinformatics (used with the permission from the copyright holder).

Canine parainfluenza virus (CPIV) belongs to the family *Paramyxoviridae* that includes simian virus 5 (SV-5), canine distemper virus, human measles, and mumps viruses. These viruses have a close antigenic relationship thus so-called *SV-5* like viruses (Ajiki et al. 1982 see Buonavogia & Martella 2007). Paramyxoviruses are pleomorphic, enveloped, and have a 150nm diameter (Quinn et al. 2011). Replication processes occur in the cytoplasm and the virions are released at the sites containing virus envelop protein. The virions are labile and sensitive to lipid solvents,

non-ionic detergents, and disinfectants. Paramyxoviruses do not exhibit recombination thus these are genetically stable and antigenic variation may occur through mutation (Quinn et al. 2011).

Infection caused by CPIV is typically restricted to the upper respiratory tract in dogs because it does not replicate in macrophages. Older animals and those that are 2 weeks of age are susceptible because their immunity levels are low (Appel & Binn 1987 see Buonavogia & Martella 2007). The mode of transmission occurs mostly by aerosolized microdroplets, and the incubation period is between 2-8 days. Virus shedding can occur 8-10 days after infection (Appel & Binn 1987 see Buonavogia & Martella 2007).

Throughout the world, CPIV is considered one of the common pathogens of highly contagious acute onset of cough in dogs (Decaro et al. 2016). Viremia is uncommon (Ford 2012). In most cases, a serious nasal discharge can be seen, and inflammation of tonsils can develop (Ford 2012).

2.7 Bacteria and Mycoplasmas species

B. bronchiseptica is a gram-negative bacterium and considered as one of the principal causative agents of kennel cough (Ford 2006). *B. bronchiseptica* is associated with respiratory infection in dogs and its complex mode of action has been reviewed (Musser 1987), for example, fimbriae (hair-like appendages extending from the cell membrane of *B. bronchiseptica* that recognize specific receptors within the respiratory tract). This aids *B. bronchiseptica* to colonize the specific tissues. Most of the time the mode of transmission is direct contact or with aerosolized microdroplets from sick dogs. The duration of incubation is 6 days (Ford 2006). *B. bronchiseptica* has a unique characteristic to invade host cells although it is an extracellular pathogen (Ford 2006).

Mycoplasmas species are gram-negative, optional aerobic bacteria that are different because it is lacking a cell wall (Bemis 1992). Mycoplasmas are a known causative agent in a dog's upper respiratory tract infection, but its role in CIRD is not completely clear. Previously, it was considered as the normal flora when the bacterium was isolated from healthy individuals (Rosendal 1978; Englund et al. 2003). *Mycoplasma cynos* is a part of CIRD and is well-screened (Rosendal 1978). Moreover, Chalker et al. (2004) studied different species of *mycoplasma* in dogs having clinical signs and no clinical signs. Samples were taken from the trachea, tonsils, and lungs. In the study, *mycoplasma* species were identified by PCR. It also showed that *mycoplasma cynos* were associated with significant respiratory disease (Chalker et al. 2004).

2.8 Canine adenovirus type 2 (CAV-2)

Adenovirus derived from (Greek word *adenos,* gland) is icosahedral 70 to 90 nm in diameter, first isolated from explant cultures of human adenoids (Quinn et al. 2011). Non-enveloped, having double-stranded DNA viruses. Adenovirus forms intranuclear inclusion in bodies and replicates in the nuclei. It is moderately stable in the environment. Adenoviruses belong to the family *Adenoviridae* (Quinn et al. 2011).

The mode of the transmission of canine adenovirus type 2 (CAV-2) is oronasal contact and the virus replicates in the epithelium of nasal mucosa (Buonavoglia & Martella 2007). The peak time of replication is between 3 to 6 days after inoculation. (Buonavoglia & Martella 2007). CAV-2 infection is most of the time associated with interstitial pneumonia (Appel 1987 see Buonavoglia & Martella 2007).

2.9 Calicivirus

2.9.1 Structure of calicivirus

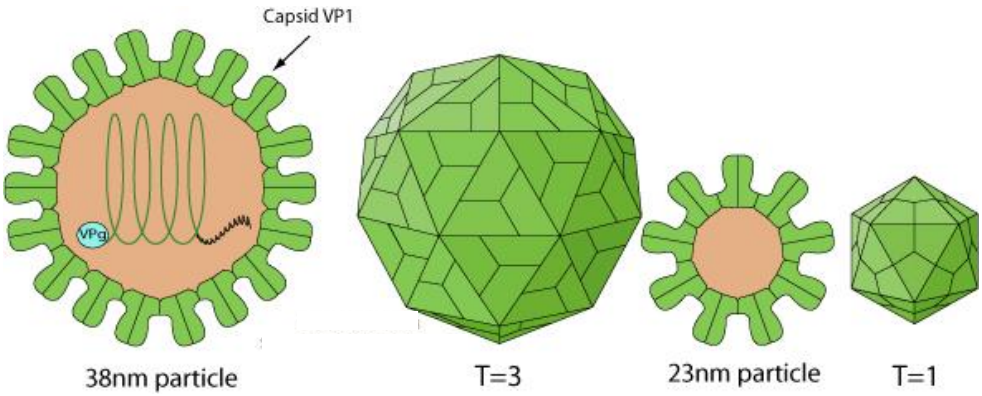

Figure 2. Structure of Calicivirus virion. Illustrated from Swiss Institute of Bioinformatics (used with the permission from the copyright holder).

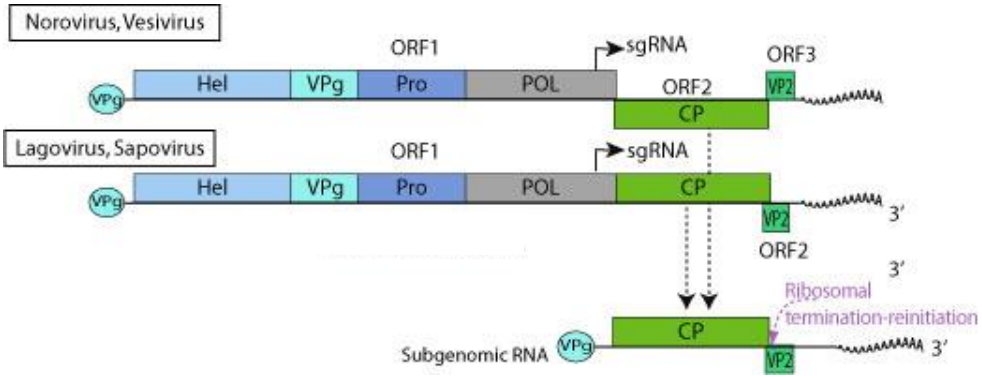

Figure 3. Genome structure of calicivirus. Illustrated from Swiss Institute of Bioinformatics (used with the permission from the copyright holder).

Caliciviruses have great significance in both veterinary medicine and human medicine (Clarke et al. 1997; Green 1997). Caliciviruses family (*Caliciviridae*) are positive sense, non-enveloped, single-stranded RNA viruses (Quinn et al. 2011). Replication occurs in the cytoplasm of infected cells, and virions are released by cell lysis. The virions of calicivirus are resistant to chloroform, ether, and mild detergents (Quinn et al. 2011). Family *Caliciviridae* consists of five genera: namely *Vesivirus*, *Lagovirus*, *Sapovirus*, *Norovirus*, *Nebovirus* with many additional candidate genera or species suggested by the International Committee on Taxonomy of Viruses (ICTV) (Clarke et al. 2012). Feline calicivirus (FCV), belonging to the genus *Vesivirus*, is a well-known veterinary pathogen and causes serious respiratory infection in cats despite vaccination practices (Dawson & Willoughby 1999). In the *Vesivirus* two species currently approved by ICTV are vesicular exanthema of swine (VESV) and feline calicivirus (FCV) (Binn et al. 2018). Many canine caliciviruses have a close resemblance to the feline calicivirus (FCV) and vesicular exanthema of swine virus (VESV) in cell culture specificities, serology, and phylogeny (Binn et al. 2018).

Calicivirus has been recovered from many species including man, cattle, dogs, cats, pigs, marine mammals, reptiles, amphibians, and insects, and linked to a wide range of diseases including respiratory disease, vesicular lesions, gastroenteritis, and necrotizing hepatitis (Quinn et al. 2011).

2.10 Canine calicivirus

In literature, very little information is found about calicivirus infection in domestic dogs. There have been occasional reports suggesting that the dogs infected with the calicivirus have acquired it from the cat's feline calicivirus (Crandell 1988). In some research, it has also been found that dogs caliciviruses, which cause enteric

infections, glossitis, vesicular genital disease or diarrhea in dogs, shared similarity to feline (FCV) antigenically (Evermann et al. 1985; Crandell 1988; San Gabriel et al. 1996). The following previous studies show the cases of canine calicivirus in different countries.

In 1985, in the United States, CaCV was identified in the feces of a four-year-old Nashville dog with bloody diarrhea and central nervous system disorder (Schaffer et al. 1985). A rectal swab was used as a specimen. In the sample CPE (Cytopathic effects) was observed by 48 hours and no other virus was isolated from the sample. The dog could not survive, so the direct cause of death was unknown because post-mortem was not performed. Parvovirus causes bloody diarrhea, and it was ruled out because this dog was negative against parvovirus, and no specific IgM was found for parvovirus. It had also been noted that four other dogs that were sharing the same household were healthy for one month before and after the death of the first dog. Virus isolations were not performed in these contacted healthy dogs, but serum samples showed specific antibodies against CaCV. It was shown to be canine calicivirus and shown to be unrelated to the feline calicivirus (Schaffer et al. 1985). The same study attempted to perform experimental infection of canine calicivirus (CaCV) in four puppies, where the oral and intranasal dose of CaCV was given. The puppies were observed for 28 days on daily basis for clinical signs of diseases including diarrhea, and their blood samples were taken on days 0, 14, and 28 for measuring antibodies against CaCV. The result of this experimental infection shows no clinical signs in puppies, but it was observed that puppies developed transitory IgM and prolonged antibody titers suggesting *in vivo* viral replication (Schaffer et al. 1985).

Mochizuki et al. (2002) performed research in Japan during the period from (January 2000 to September 2001), 14 oral swabs samples and 105 fecal samples were obtained in different locations from private animal hospitals. The results show two puppies were found to be positive for canine *vesivirus*. One of these, a 2-month-old pup having clinical signs of upper respiratory tract infection and diarrhea (Mochizuki et al. 2002). The sample was taken from a tonsil swab of the puppy. The second of these puppies, aged 50 days showed clinical signs of diarrhea. The rectal swab was used as the sample in the second puppy. These two puppies were negative against canine parvovirus type-2, canine distemper virus, canine coronavirus, canine adenovirus, and rotavirus. However, PCR amplification performed on both samples yielded a 331bp size PCR product which suggested that the virus was either *vesivirus* or *Sapporo like virus*. The sequence result indicated that (97.9% to 98.9%) identity with strain 48. The strain 48 is classified as a novel member of *Vesivirus* genus. This strain was also unrelated to feline calicivirus, and on this tentative basis, it was proposed as “true” CaCV and was included in the genus *Vesivirus* (Matsuura

2002; Roerink 1999). Antibodies against CaCV strain 48 was detected in 57 percent of dogs in Japan (Mochizuki et al. 2002).

Jang et al. (2003) researched the seroprevalence of canine calicivirus in the Republic of Korea. In the study, sera were collected from 319 domestic dogs that displayed various health problems. The CaCV number 48 strain (Mochizuki et al. 1993) was used as a reference. Neutralizing anti-body against CaCV was detected in 116 dogs (36.5%). This finding proves that CaCV infection present in Korean dogs.

In Brazil, Custo et al. (2003) detected the canine calicivirus in 01 out of 17 clinical samples. This sample that tested positive for canine calicivirus was also positive for canine coronavirus. The objective of Custo et al, (2003) study was to find CaCV association with diarrhea from puppies.

Martella et al. (2008) identified the novel calicivirus from a 2-month-old pup with clinical signs of diarrhea and vomiting. The pup recovered completely after 4 days. But during hospitalization, the feces were screened for common viral pathogens. This pup was also positive for canine parvovirus type-2a (CPV-2a). The pup was kept under observation after detection of viruses and its fecal sample was monitored for 30 days on daily basis. It had been observed that canine parvovirus-2a was found until 10 days of post-hospitalization while calicivirus was detected until 22 days. This isolate was genetically related to the lion norovirus strain having 90.1% amino acid identity in the capsid protein.

Mesquita et al. (2010) found canine norovirus in dogs. In the study total of 105 fecal samples were collected in 3 districts in Portugal. The fecal samples were collected from veterinary clinics, dog shelters, and pet shops. In the study, 63 samples from dogs with diarrhea and 42 samples from normal feces (control group) were used. Reverse transcription- PCR used for diagnosis of the virus. The results showed that canine norovirus was detected in 40% of dogs with diarrhea and 9% without diarrhea (control group) respectively.

Ntafis et al, (2010) conducted a pilot study in Greece. The samples were collected from kennels, pet shops, and dogs that were hospitalized in veterinary clinics showing gastroenteric symptoms. In the study total of 72 fecal samples were used from 2- to 8-month dogs. The results showed that 6 of 72 (8.3%) tested positive for norovirus. These 6 norovirus-positive dogs showed clinical signs of diarrhea, and all were co-infected with canine coronavirus.

Binn et al. (2018) conducted a study at the Walter Reed Army Institute of Research (WRAIR) in 1963-1978, where four unidentified viruses were recovered in addition to many other known canine viral pathogens. These four unidentified viruses produced similar cytopathic effects (CPE). Observations based on the electron microscopy imaging and physicochemical studies suggested that these isolates were likely calicivirus having similar characteristics to other caliciviruses, for example,

feline calicivirus (FCV), vesicular exanthema of swine virus (VESV), and Norwalk like virus (NVL, norovirus). Genome sequencing of these 4 viruses was conducted in 2016 and the sequence results identified them as *Vesivirus* and their genetic relationship to another member of *calicivirae* family. These 4 viruses are designated as 3-68, L198T, A128T, and W191R. These 4 viruses were recovered from the respiratory sample (two of them), from fecal (one of them), and penile sample (one of them) respectively. Among the 4 dogs, one died shortly after collection of samples, the remaining 3 dogs had a co-infection, the dog designated as (L198T) co-infected with canine coronavirus, the second dog (A128T) co-infected with canine picornavirus, and third dogs (W191R) co-infected with canine parainfluenza virus, these indicating a case of mixed infection (Binn et al. 2018).

Renshaw et al. (2019) found in their study that four out of eleven dogs affected with hemorrhagic diarrhea and vomiting died within 24 to 72 hours despite immediate treatment. Routine diagnosis procedure could not find the specific causes of the death but in *situ* hybridization and reverse transcription PCR assay confirmed the presence of canine *vesivirus* from fresh necropsy tissue of deceased dogs. Renshaw et al. (2019) also noticed that hemorrhagic gastroenteritis and vomiting were common in eleven affected dogs, but the respiratory disease was not found to be of concern in any of the eleven affected dogs. In their study, canine *vesivirus* (CaVV) was localized in endothelial cells of arterial and capillary blood vessels (Renshaw et al. 2019).

3 Material and methods

3.1 Animals

The samples were collected from 2013-2016, throughout Sweden. All dogs were sampled using nasal-oral swabs. In this study, a total of 75 privately own dogs screened in which 59 dogs with CIRD and as controls 16 healthy dogs were included. The 16 healthy dogs that used in the study had not suffered from respiratory signs for the last six months.

Samples were collected up to one week from on-set of characteristic clinical signs of kennel cough. All breeds, gender and ages of dogs were included in the study. Samples were collected from different veterinary clinics throughout Sweden with the permission of the dog owners. No more than two dogs per household were sampled. Information about the complete history from each dog including vaccination status, duration of clinical signs, and dog activities, were collected.

The clinical signs of CIRD were categorized in three main categories: mild, moderate and severe signs. In mild signs, the dog coughed at irregular intervals. In the moderate type, the dog suffered from a strong and persistent cough. The severe category depicts dogs that have had a severe deep cough with affected general condition.

The study was approved by local ethical committees (ID-No: C127/14 and C227/11).

3.2 RNA extraction

Nucleic acids from the nasal-oral swabs were extracted using the Magnatrix 8000+ 96-well extraction robot at SVA (National Veterinary Institute), and extractions have been stored at -80°C until further use. The samples have been screened for

causative agents like CAV-2, CIV, CPIV-2, CRCoV, *M. canis, M. cynos* and *B. bronchiseptica* in previous projects.

3.3 Primers for CaCV detection

Primers were designed based on the sequence of CaCV that was the outcome of the viral metagenomics investigation. A 411bp fragment of the conserved putative RNA dependent RNA pol region was chosen, and the primers were ordered from Sigma.

Table 1. *Primer used for PCR*

Primer	Sequence (5'-3')	Tm (°C)	Size(bp)
CaCV_det_Fw	GAGACTTTACACACCAGGAACTC	60.7	411
CaCV_det_Rev	GAGAACACCTCCGGCATAAC	63.4	411

3.4 Positive control

The positive control was a synthesized dsDNA 580bp fragment with primer binding sites and over all GC-composition identical to the expected CaCV sequence. The middle region of the sequence was inverted in order to avoid false positives. The fragment was ordered from Gene Art string (Thermo Fisher Scientific), (Lot number 2318747) and used at a final concentration of 0.7 µg/µl.

3.5 One-Step RT-PCR with ezDNase™

The SuperScript™ IV One-Step RT-PCR System with ezDNase™ (ThermoFisher Scientific) protocol was followed. RNA template of 5-8µl according to the availability of clinical sample was used. A negative control of 8µl of nuclease-free water was used.

The PCR amplification was run on Proflex TM 3X 32 well PCR system (Applied biosystem by life technologies, 4484073). The thermo profile of the one-step RT-PCR was reverse transcribed at 50°C for 10 min, initial denaturation at 98°C for 2min, followed by amplification for 40 cycles at 98°C 10sec,63.4°C for10 sec and 72°C for 30 sec, with a final extension of 75°C for 5min. In each run, the positive control (from the gene art, described above) and a negative control was added.

3.6 Gel electrophoresis

A 2% agarose gel was made using 1xTBE buffer, Universal Agarose, PeqGoldElectron (VMR Life science) and Gel Red Nucleic Acid (Biotium). The gel was loaded with 1µl of load dye mixed with 5µlof PCR product. The gel was run at 100 volts for 45 minutes. Gene Ruler 1 Kb Plus DNA ladder (Thermo Fisher Scientific) was used as a ladder. The gel was read on the ChemiDoc touch imaging system (Bio-Rad).

3.7 DNA Sequencing

Positive samples were purified by Gene JET PCR purification kit (Thermo Fisher Scientific) according to the manufacturer's instructions and sent to Macrogen Europe for Sanger sequencing. The sequences we got from the company were cleaned, trimmed and aligned to each other by using the software Codon Code. Quality sequences of four clinical samples were further used to make a phylogenetic tree.

3.8 Phylogenetic analysis

For making the phylogenetic tree, the online version (Phylogeny. fr) was used and to further improve the visualization (Interactive Tree of life (iTOL) v5: an online tool for phylogenetic tree display and annotation) was used ((Letunic & Bork 2021). Nucleotide sequences were translated into amino acids sequences to build the phylogenetic tree on the amino acids. Tree construction was performed with one outgroup, canine astrovirus isolate. GenBank accession numbers are shown in the tree.

4 Results

4.1 Detection of canine calicivirus

In this project, dogs with CIRD (n=59) and healthy dogs (n=16) were included. In total 75 dogs have been used in this study. Out of the 75 samples screened for canine calicivirus, 7 were found to be positive. All the dog's samples (n=7) that were positive for canine calicivirus were only from sick dogs. No sample was observed positive in healthy control. No statistically significance relationship was seen between CIRD and canine calicivirus (Fishers exact test; P = 0.33) (Table 2).

Four dogs that were found positive for canine calicivirus were adults (1-8 years old), while one was a 3-month-old puppy, no information of age for remaining two dogs (Table 3).

Table 2. *Prevalence of canine calicivirus in sampled dogs*

Pathogen	Dogs with CIRD n =59	Healthy dogs n =16	P-value
CaCV	7 (11.86%) 95% CI,0.04-0.22	0 (0%) 95% CI,0.0-0.0	0.33

CaCV, canine calicivirus; CIRD, canine infectious respiratory disease; 95% CI, confidence interval.

Table 3. *Main findings of Canine Calicivirus (CaCV) positive samples in study*

	Sample no	Sampling site	Sampling date	Age Years
CaCV1	9295	Västerås	May 2013	2
CaCV2	0143	Helsingborg	June 2013	7
CaCV3	5831	Helsingborg	Nov 2013	8
CaCV4	1403	Helsingborg	Jan 2014	1
CaCV5	6153	Klavreström	*	*
CaCV6	6157	Klavreström	*	*
CaCV7	6902	Albano	Jan 2014	0.33

CaCV, canine calicivirus; *, no information.

4.1.1 Co-infection

The clinical samples analysed in this project were previously screened for different causative agents like CAV-2, CIV, CPIV-2, CRCoV, *M. canis*, *M. cynos* and *B. bronchiseptica.* The main finding in prevalence of CPIV-2 in clinical samples was 29 dogs although CRCoV was found in 13 dogs and *M. cynos* total positives were 15 while 76 numbers of positive samples were seen for *M. canis*. In relation to previous screened pathogens, no co-infection was seen in two dogs. Another two dogs had a co-infection only with *M. canis.* One of the dogs had co-infection with CRCoV, *M. canis* and *M. cynos*. In remaining two dogs had a co-infection with CPIV-2 and *M. canis*. Table 4 shows the detail of co-infection of canine calicivirus.

Table 4. *Shows the co-infection of canine calicivirus.*

	Sample number in study	Co-infection
CaCV 1	9295	*M. canis*
CaCV 2	0143	No co-infection
CaCV 3	5831	No co-infection
CaCV 4	1403	*M. canis*
CaCV 5	6153	CPIV-2+ *M. canis*
CaCV 6	6157	CPIV-2+ *M. canis*
CaCV 7	6902	CRCoV+ *M. canis* +*M. cynos*

CaCV, canine calicivirus; *M. canis*, *Mycoplasma canis; M. cynose*, Mycoplasma *cynose*; CRCoV, canine respiratory coronavirus; CPIV-2, canine parainfluenza virus type-2

4.1.2 Negative for various pathogens

Canine calicivirus positive samples were also checked for different pathogens in previous studies. Molecular diagnostic (real-time/RT- PCR) were performed in previous studies for CAV-2, CHV, CIV, CRCoV, *B. bronchiceptica*, *M canis*, *M cynos*, and CnPnV. The results in table 5 show that 7 samples that found positive for CaCV found negative for various pathogens.

Table 5. *Shows the seven canine calicivirus samples in this study consider negative for previously screened pathogens.*

	Sample number in study	Consider negative
CaCV 1	9295	CAV-2, CHV, CIV, CPIV-2, CRCoV, *B. bronchiseptica*, *M.cynos*, CnPnV.
CaCV 2	0143	CAV-2, CHV, CIV, CPIV-2, CRCoV, *B. bronchiseptica*, *M. canis*, *M. cynos*, CnPnV.
CaCV 3	5831	CAV-2, CHV, CIV, CPIV-2, CRCoV, *B. bronchiseptica*, *M. cynos*, *M. canis*, CnPnV.
CaCV 4	1403	CAV-2, CHV, CIV, CPIV-2, CRCoV, *B. bronchiseptica*, *M. cynos*, CnPnV.
CaCV 5	6153	CAV-2, CHV, CIV, CRCoV, *B. bronchiseptica*, *M. cynos*, CnPnV.
CaCV 6	6157	CAV-2, CHV, CIV, CRCoV, *B. bronchiseptica*, *M cynos*, CnPnV.
CaCV 7	6902	CAV-2, CHV, CIV, CPIV-2, *B. bronchiseptica*, CnPnV.

CAV-2, canine adenovirus type-2; CHV, canine herpesvirus; CIV, canine influenzavirus; CPIV-2, canine parainfluenza virus type-2; CRCoV canine respiratory corona virus; *B. bronchiseptica*, *Bordetella bronchiseptica*; *M. cynos*, *Mycoplasma cynos*; *M. canis*, *Mycoplasma canis*; CnPnV, Pneumovirus

4.1.3 Grading of clinical signs

Dogs displaying clinical signs of kennel cough were grouped as having mild, moderate and severe signs based on observed symptoms. The dogs that found positive for canine calicivirus 6 out of 42 (14%) from mild clinical signs and 1 out of 11(9%) found positive from moderate infection. In severe form 0 out of 6 (0%) considered positive for CaCV and from healthy control 0 out of 16 (0%) tested positive (Table 6).

Table 6. *Detail of results based on the clinical signs compared with healthy controls.*

Clinical signs	CaCV+	CaCV-	Total
Healthy	0	16	16
Mild	06	36	42
Moderate	01	10	11
Severe	0	06	06

CaCV+, canine calicivirus positive; CaCV-, canine calicivirus negative
Healthy – no upper respiratory signs for at least 6 months; Mild – only mild intermittent coughing.
Moderate – severe frequent cough without affected general condition; Severe – affected general condition and deep coughing.

4.1.4 Disease duration

The dogs that found positive for canine calicivirus were sampled from day 1 to 7 days of infection. In the first day of clinical sign 1 out of 18 (5.5%) found positive for CaCV while on the second day 3 out of 13 (23%) found positive, third days of clinical signs 1 out of 10 (10%) found positive, 0 out of 7 (0%) dog found positive on fourth day of clinical signs, 1 out of 6 (16%) found positive on fifth day, 1 out of 3 (33%) found positive on day six of infection and 0 out of 2 (0%) dogs found positive on day seven of infection (Table 7).

Table 7. *Distribution of molecular diagnostic result of canine calicivirus in relation to duration of signs*

Duration of signs (days)	CaCV+	CaCV-	Total
1	1	17	18
2	3	10	13
3	1	9	10
4	0	7	7
5	1	5	6
6	1	2	3
7	0	2	2

CaCV+, canine calicivirus positive; CaCV-, canine calicivirus negative.

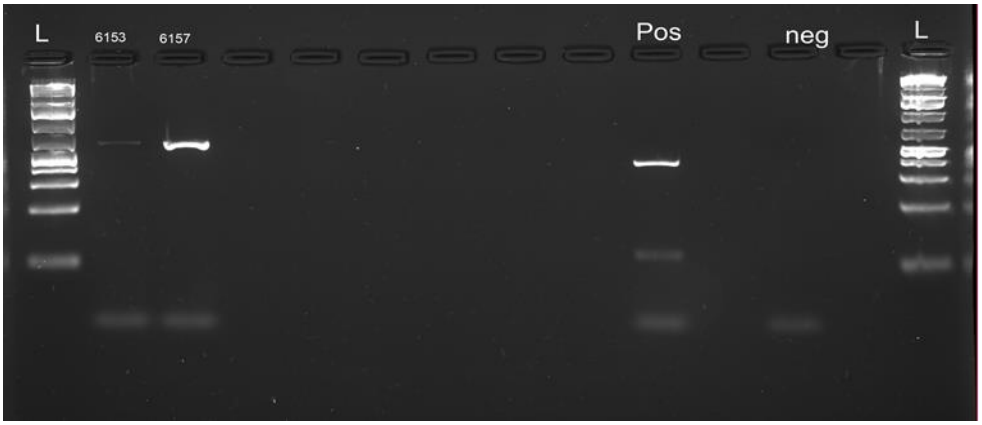

Figure 4. One example of gel results in which the samples with ID 6157 clearly shows positive for canine calicivirus, and ID 6153 is weakly positive. L, 1 Kb Plus DNA ladder; Pos, Positive control; neg, negative control.

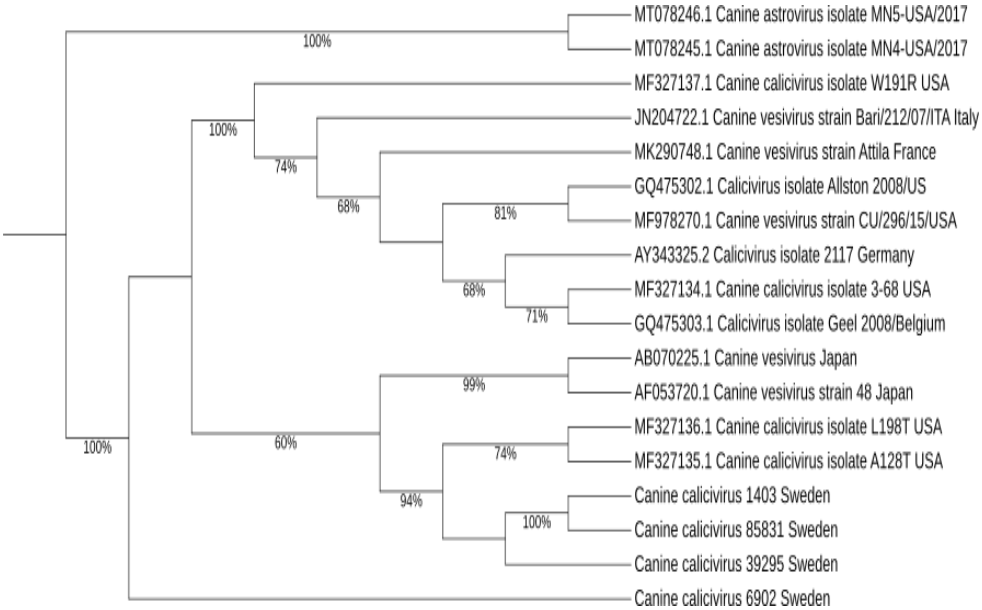

Figure 5. Phylogenetic tree based on the nucleotide sequences of the RNA-dependent RNA polymerase (RdRp).

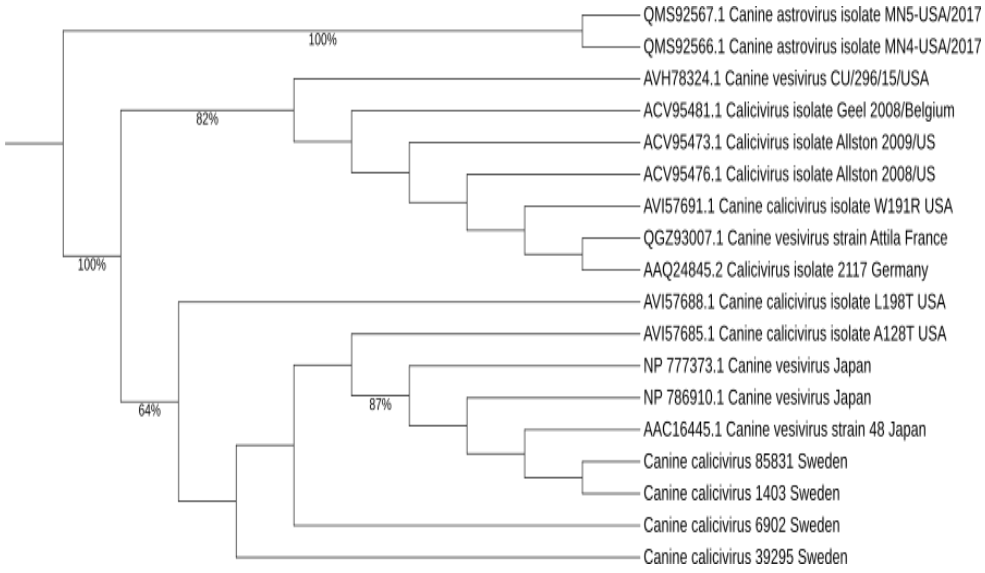

Figure 6. Phylogenetic tree based on amino acid sequences of the RNA-dependent RNA polymerase (RdRp).

5 Discussion

Thus, the aim of this study was to check how many samples were positive for canine calicivirus and to find any possible association between canine calicivirus and CIRD.

In this study, oronasal swabs have been used for the detection of the canine calicivirus. In previous research, Binn et al. (2018) isolated the canine calicivirus from a throat swab of a dog. However, in other countries in Greece, Portugal, Brazil (Ntafis et al. 2010; Mesquita et al. 2010; Castro et al. 2013) fecal samples have been used to detect the virus in dogs.

To investigate the prevalence of canine calicivirus in Swedish dogs with CIRD and healthy control, a one-step RT-PCR assay was developed and used in this project. A classic RT approach was performed because the objective was to send the positive samples for Sanger sequences to retrieve longer stretches of viral genome for sequence analysis. In previous studies in Japan (Mochizuki et al. 2002), Brazil (Custo et al. 2003), Portugal (Mesquita et al. 2010), this same approach RT-PCR was used to detect the canine calicivirus.

In this study, canine calicivirus were found in a total of 9.3% (7/75) of dogs tested. The prevalence of CaCV was 11.86% (7/59) for dogs with CIRD and interestingly none of the dogs (0/16) belonging to the healthy control were tested positive.

In this study, the two dogs that tested positive for canine calicivirus did not have co-infection with previously scanned pathogens i.e., CAV-2, CHV, CIV, CRCoV, *B. bronchiceptica*, *M canis*, *M cynos* and CnPnV as described in table (7) in the result section. Although two dogs tested positive only for *M. canis* and two other dogs were found to be co-infected with CPIV-2 and *M. canis*. One remaining dog had a co-infection with CRCoV, *M. canis* and *M. cynos*. In previous studies, co-infections of canine calicivirus and coronavirus have been observed. In Greece, Nfafis et al. (2010) detected Norovirus 6 of 72 (8.3%) and all were coinfected with canine coronavirus. In Brazil, Custo et al. (2003) detected the canine calicivirus in 01 out of 17 clinical samples. This sample that tested positive for canine calicivirus

was also positive for canine coronavirus. Binn et al. (2018) recovered four canine calicivirus from military dogs and one of the dogs co-infected with canine respiratory coronavirus.

In this study, the dogs diagnosed for CaCV were grouped, based on clinical symptoms i.e., mild (only mild intermittent coughing), moderate (severe frequent cough without affected general condition), and severe (affected general condition and deep coughing) as described in the results. The dogs that were found positive for canine caliciviruses 6/42 (14%) from mild, 1/11(9%) from moderate, 0/6 (0%) from severe were also positive for canine calicivirus. However, to date, no previous study for CaCV has found that categorises the CIRD based on mild, moderate, and severe clinical infection in dogs.

Phylogenetic analysis showed that the four Swedish canine caliciviruses that were used in the tree were genetic divergent. The possible explanation for this genetic diversity could be that different dog owners travel with their dogs to different countries and then are exposed to viral infections. Another possible reason could be that the clinical samples were collected from different veterinary clinics from household dogs. In those veterinary clinics many clients from different nationalities visit the clinics with their dogs, some clients have already travelled to different countries with their dogs. Viral infection can then spread from one dog to another. Another explanation could be that the genetic variation shown here, is what can be expected as these are viruses with high mutation frequencies. Our findings are consistent to Binn et al. (2018), who reported high genetic diversity in canine calicivirus samples from the USA.

Results from this present study reveal that the canine calicivirus is present in dogs with canine respiratory disease in Sweden. However, to confirm CaCV association with CIRD and its pathogenicity in dogs more studies are needed such as experimental infection of CaCV in pathogen-free dogs can be helpful to get more information about pathogenicity in dogs.

The results indicating positive cases of canine calicivirus in this study also suggest a further need to study the seroepidemiology of the CaCV especially in those areas of Sweden where many dogs meet e.g., dog daycare centers, dog shows, animal shelters, kenneled dogs, etc. In this present study, samples were collected from Swedish household dogs however, serum samples collected from other areas where a group of dogs interacts their serum samples provide a broader view to understand the prevalence of CaCV.

6 Conclusion

This is the first description of canine calicivirus in dogs with CIRD and to date, it is also to our knowledge the first description of canine calicivirus in Sweden. The overall, prevalence of canine calicivirus in this study was 11.86% with CIRD whereas no virus was detected in the healthy control group. No statistically significant association was found between the presence of canine calicivirus and kennel cough, but this could be due to a very small sample set. Out of 7 canine calicivirus positive dogs, no coinfection has been found in two dogs, however, another two dogs were coinfected with *M.canis*, while in the remaining three dogs mixed infection has been found. Our study results indicate that canine calicivirus exists in Swedish household dogs that show CIRD infection. However, there is a need for further research on canine calicivirus to understand the clinical relevance in kennel cough. Genetic characterization of the complete virus genome is also needed.

7 Summary

Canine infectious respiratory disease (CIRD) is an acute, highly contagious disease complex caused by many causative agents both bacteria and viruses. Some of the causative agents have been diagnosed in previous research and vaccines are also available in the market for some of the common agents (CPIV-2 and *B. bronchiseptica*), but still outbreaks of kennel cough are recorded globally. It could be because some of the causative agents involved in kennel cough are still not known. Before starting this project, a metagenomic study was performed on 14 dogs in which 12 dogs had CIRD and 2 healthy dogs were used. According to the metagenomic study results, 2 out of the 12 dogs with CIRD tested tentative positive for canine calicivirus according to sequence results, one of which only tested positive for canine calicivirus with no other previous scanned pathogens. Another reason is to find the canine calicivirus role in CIRD because previous studies show that calicivirus causes respiratory disease in cats. Thus, the aim of this study is to find canine calicivirus association with CIRD. According to the best of our knowledge, to date, this is the first study in which canine calicivirus has been found in Swedish household dogs with CIRD and describes canine calicivirus possible association with CIRD in Sweden.

In this study, samples were collected from Swedish household dogs from veterinary clinics throughout Sweden. In total n=75 dogs were used in which n=59 dogs with CIRD, n=16 healthy dogs were used. The results finding shows that n=07 dogs tested positive for canine calicivirus and none of the dogs tested positive from a healthy control group. Statistical analysis shows data is insignificant (Fishers exact test; P = 0.33). In total n=59 dogs with CIRD were used in the study and dogs were graded into three categories: mild, moderate, and severe signs. The dogs that tested positive for canine calicivirus 6 out of 42 (14%) with mild signs, 1 out of 11 (09%) tested positive for canine calicivirus from moderate signs, 0 out of 6 (0%) dogs tested positive for canine calicivirus from severe signs. No dog tested positive calicivirus from healthy control out of 16 dogs this might be due to a smaller number of samples used as healthy control. The 07 clinical samples that were tested positive

for canine calicivirus in the present study have also been checked for the co-infection with previously scanned pathogens CAV-2, CIV, CPIV-2, CRCoV, *M. canis*, *M. cynos* and *B. bronchiseptica*. No co-infection has been found in two dogs. Another two dogs had co-infection only with *M. canis*. One dog had co-infection with CRCoV, *M. canis* and *M. cynos*. In the remaining two dogs, co-infection was seen for CPIV-2 and *M. canis*. However, the results from the present study show that canine calicivirus exists in Swedish household dogs that show CIRD infection.

To understand more about the pathogenicity of canine calicivirus and its association with CIRD more research is needed such as experimental infection in pathogen-free dogs. An advanced study is also needed to understand the canine calicivirus complete genome, genetic diversity, and its seroepidemiology that will help to develop the vaccine, to stop infection, and to provide adequate advice to dog owners.

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