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A serological and virological investigation into the prevalence of enteric and respiratory coronaviruses in the Hungarian and Austrian dog population.

Thesis work

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2. List of Abbreviations

BoCoV	=	Bovine coronavirus
BLAST	=	Basic local alignment search tool
CAD	=	Canine adenovirus
CCoV	=	Canine coronavirus
CDV	=	Canine distemper virus
CECoV	=	Canine enteric coronavirus
CHV	=	Canine herpes virus
CIRD	=	Canine infectious respiratory disease
CIV	=	Canine influenza virus
CPE	=	Cytopathic effects
CPIV	=	Canine parainfluenza virus
CRCoV	=	Canine respiratory coronavirus
DNA	=	Deoxyribose nucleic acid
ELISA	=	Enzyme-linked immunosorbent assay
FCWF	=	Felis cati whole foetus
FeCoV	=	Feline coronavirus
FECoV	=	Feline enteric coronavirus
FFPE	=	Formalin-fixed, paraffin-embedded
FIPV	=	Feline infectious peritonitis
FITC	=	Fluorescein isothiocyanate
HCoV	=	Human coronavirus
HECoV	=	Human enteric coronavirus
IFA	=	Immune fluorescence dose
MDBK	=	Madin-Darby bovine kidney
MEM	=	Minimal effective medium
OIE	=	World Organisation for Animal Health (Office International des Epizooties)
PBS	=	Phosphate buffered saline
PRCoV	=	Porcine respiratory coronavirus
RNA	=	Ribonucleic acid
RT-PCR	=	Reverse transcription polymerase chain reaction
SARS	=	Severe acute respiratory syndrome

TCID	=	Tissue culture infectious dose
TGEV	=	Transmissible gastroenteritis virus
UTR	=	Untranslated region
VN	=	Virus neutralisation

3. Introduction

Coronavirus infections are a worldwide problem of domestic dogs with significant health and economic importance. Coronavirus infections in dogs have two forms; an enteric form caused by Canine enteric coronavirus (CECoV I and CECoV II) and a respiratory form caused by canine respiratory coronavirus (CRCoV). Many privately owned dogs are currently vaccinated for kennel cough (a poly-aetiological disease involving CRCoV) but the necessity to vaccinate is based on the prevalence of the disease and the likelihood for the animal to be exposed to other dogs. A vaccine for the enteric form of coronavirus infection is also available but is not commonly administered to dogs. The decision to vaccinate for both forms of coronavirus infection must be based on a risk-benefit analysis by the veterinarian and the prevalence of the disease is the most important factor in deciding on the correct course of action.

The aim of this study was to survey and quantify the prevalence of both enteric and respiratory forms of Coronavirus infection in the Hungarian dog population. Although the presence of CECoV and CRCoV in Hungary has previously been demonstrated (Lakatos et al., 2013) there were no targeted surveys of the occurrence of CECoV and CRCoV. To accomplish this aim a large number of samples were taken from the Hungarian shelter and privately owned dog populations and the samples were analysed using a mixture of direct and indirect laboratory diagnostic methods.

The study was part of a partner project with the University of Veterinary Medicine, Vienna, Austria who performed the same survey on the Austrian dog population (Spiss et al, 2012). The study was carried out on privately owned dogs and shelter dogs that had not been previously vaccinated for coronavirus. Serum samples were taken from dogs exhibiting no clinical signs of coronavirus infection and investigated using virus neutralisation and indirect immune fluorescence tests. The use of indirect serology methods to detect the presence of the virus allowed for a long range view of the prevalence of the virus in Hungarian population as antibodies persist for a long time in the serum after exposure to the virus.

Faecal samples and naso-pharyngeal swabs were collected from dogs exhibiting symptomatic signs of coronavirus infection. Lung and intestinal tissue samples were taken from dogs which

had recently succumbed to a disease with symptoms similar to those of a coronavirus infection. These samples were analysed with reverse transcription polymerase chain reaction (RT-PCR) assay and positive samples were further analysed by partial nucleotide sequence determination to differentiate between different canine coronavirus serotypes. The epidemiological survey consisted of four distinct test groups;

1. Study 1: Serological survey of the prevalence of CECoV infection in Hungarian dogs.
2. Study 2: Detection of CECoV in clinically ill and recently deceased Hungarian dogs.
3. Study 3: Serological survey of the prevalence of CRCoV infection in Hungarian dogs.
4. Study 4: Detection of CRCoV in clinically ill and recently deceased Hungarian and Austrian dogs.

4. Literary Review

4.1 Morphology and Taxonomy of coronaviruses

Coronaviridae is a family of large, pleomorphic, enveloped ssRNA viruses. They attach to cells via a glycol protein called the spike protein (S protein) projecting from the surface of the envelope allowing fusion between the host cell membrane and the viral envelope (Quinn et al, 2011). This S protein is the main antigenic component of the virus and induces the production of neutralising antibodies during natural infection. Its hypervariable domain allows the virus to evade the immune response by producing virus escape mutants. Coronaviruses were first described in dogs with gastroenteritis (Binn et al., 1974) but the CRCoV antigenic strain was later determined as a distinctly different serotype to the previously known canine coronaviruses (CCoV). CRCoV showed only 69% nucleotide identity in the highly conserved polymerase region with only 21% amino acid sequence identity in the S protein, indicating CRCoV was a novel coronavirus of dogs (Erles et al., 2006).

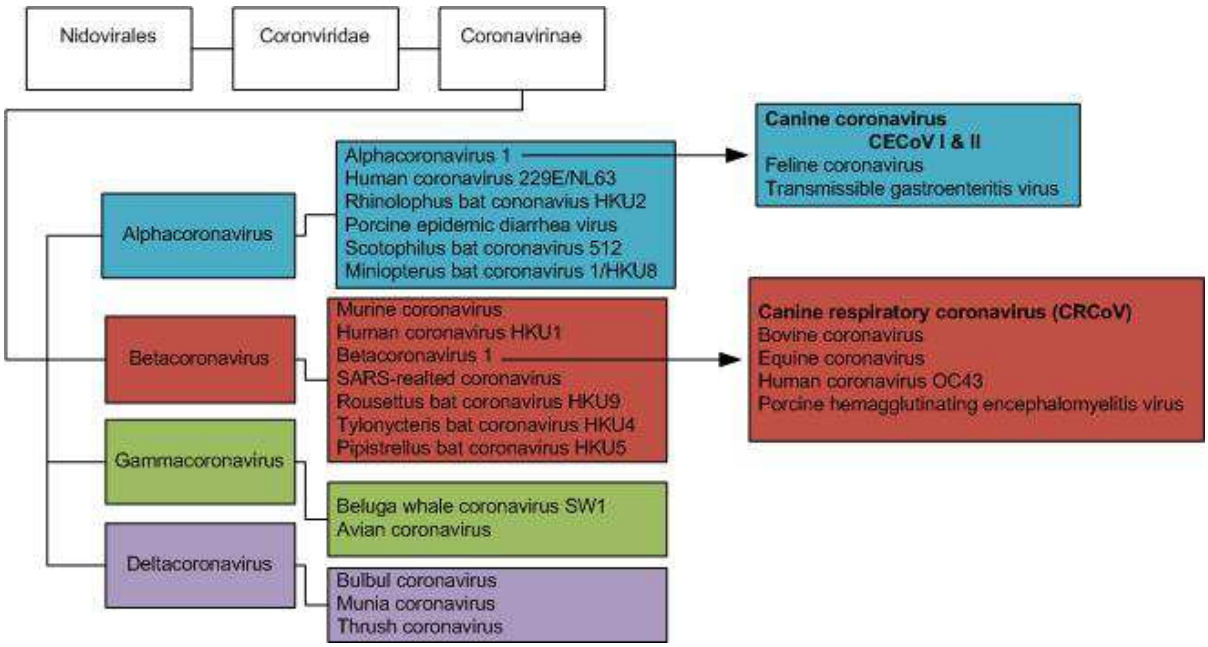

Figure 1: Taxonomy of canine enteric and respiratory coronaviruses.

Coronaviruses can infect a number of mammalian and avian species and generally display a tropism for enteric and respiratory epithelium. Taxonomically the *Coronaviridae* family is divided into 2 subfamilies; *Coronavirinae* and *Torovirinae*. The *Coronavirinae* subfamily is

subsequently divided into 4 genera; *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus* which is the most recently classified genus (Figure 1.).

Members of the *Coronavirinae* subfamily are separated into antigenic groups based on their genetic similarities. CECoV (CECoV I and II) fall into the first antigenic group including FIPV, FECoV, TGEV and PRCoV. CRCoV falls into the second antigenic group including BoCoV, HECoV and SARS. Most members of this second group are characterised by the presence of an additional gene coding for a surface hemagglutinin-esterase protein resulting in their ability for hemagglutination.

The ability of canine coronavirus to cause disease is variable and generally susceptibility decreases with age. CECoV was first isolated by from faecal samples of diarrhoeic military dogs in Germany (Binn et al., 1974). Different CECoV genotypes were isolated in Italy (Pratelli et al., 2001, 2002b, 2002c). These variants showed variation of the sequence in the gene encoding for the M-protein with similarity to FeCoV type 1. This led to the division of CECoV into two different genotypes; CECoV type 1 which is genetically very similar to FCoV type 1 in the S-gene sequence and CECoV type 2 which is similar to FeCoV type 2 (Partelli et al., 2003).

A highly virulent and fatal variant of CECoV was described in puppies following an outbreak of canine parvovirus infection (Pratelli et al. 1999) as well as a more recently described pantropic form of coronavirus which spreads in the internal organs and caused fatal infection in dogs (Buonavoglia et al., 2006). In general CECoV infections are mild and dogs recover spontaneously after 7-10 days, shedding the virus in their faeces for a further 6-14 days after infection (Keenan et al., 1976, 1979). More persistent shedding has been reported where dogs shed the virus in faeces for up to 6 months after the cessation of clinical signs (Pratelli et al., 2001, 2002c).

CRCoV is a close relative of BoCoV and HCoV-OC43 with 96% similarity in the variable S protein. The close genetic relation to BoCoV throughout the CRCoV genome indicates that the virus was probably transmitted to dogs from cattle and similarly HCoV-OC43 to humans from cattle. CRCoV is associated with mild and transient respiratory signs such as nasal discharge and persistent cough. It has been described particularly in kennelled dogs as part of canine infectious respiratory disease complex (CIRD) commonly referred to as kennel cough (Decaro and Buonavoglia, 2008).

4.2 Coronavirus disease in dogs and its importance

Coronavirus infections cause respiratory, enteric and generalised disease in both domestic and wild animals as well as humans. CCoV infection is of significant scientific interest due to its viral genome variability and its capability of asymptomatic infection of canine hosts who shed the virus. The coronavirus genome is highly variable and recombination between species specific coronaviruses have been shown or suspected (Bridgen et al., 1993; Herrewegh et al., 1998; Pratelli et al., 2002b). This ability for recombination poses a significant risk to both animal populations and public health.

The clinical manifestation of coronavirus disease in dogs is highly variable depending on the immune status of the host and the virulence of the infecting strain. They are frequently found as part of disease complexes in animals with poor immunological status kept in close confinement (Stavisky et al., 2008). Such scenarios result in endemic disease with high morbidity and low mortality.

Coronaviruses are shed in high numbers by the infected host via bodily secretions. In the case of CECoV the virus is shed via the faeces and in case of CRCoV the virus is shed via nasal discharge and saliva. The incubation period of canine coronavirus is approximately 3 days post infection and although clinical disease is reported the asymptomatic form of CCoV infections are much more prevalent (Quinn et al., 2011). The virus has relatively low resistance in the environment, requiring hosts for its maintenance (Pratelli et al., 2006). Long term carrier animals may shed the virus sporadically for a long period without demonstrating clinical signs of infection (Stavisky et al., 2008).

4.2.1 CECoV

CECoV, also refered to as alphacoronavirus 1, causes gastroenteritis in dogs. The disease was first described as a mild to severe gastroenteritis mainly in young dogs (Binn et al., 1974). The disease is characterised by vomiting, watery diarrhoea and dehydration. The infection is frequently complicated by parvovirus 2 infection causing a more pathogenic haemorrhagic enteritis with high mortality (Quinn et al., 2011). Age, immunological status and environmental factors greatly influence the severity of clinical signs in CECoV infection. Both CECoV type 1 and 2 appear to behave in a broadly similar manner clinically. Dogs of all ages can be infected but serious illness primarily occurs in pups (Quinn et al., 2011). The clinical signs of CECoV infection of pups are perfuse watery diarrhoea, severe dehydration, acidosis and vomiting. The diarrhoea typically lasts for 2-4 days and most pups survive,

developing long term immunological resistance after recovery. Untreated, weak patients may die but typically there is some other problem in the background such as parasitism, dual infections with parvovirus or malnutrition (Stavisky et al., 2008).

Inactivated vaccines are available for the protection of dogs. CECoV is shed in the faeces of infected animals and infects the new host per os. Infected dogs usually shed the virus for 9 days but infected dogs may shed CECoV intermittently for months (Quinn et al, 2011). The virus is not resistant in the environment and hosts are needed for the maintenance of the infection (Pratelli et al., 2006). CECoV is able to withstand the acidic environment of the stomach and infect enterocytes of the upper small intestine, spreading rapidly to other parts of the small intestine. Damage to the mature enterocytes at the tip of intestinal villi results in a loss of the digestive and absorptive capacity of the small intestine and watery diarrhoea as a clinical sign. The local mucosal immunity plays a more important role for protection of the dog from re-infection than the circulating antibodies. In the absence of frequent re-exposure to the virus, the duration of the immunity may be relatively short (Quinn et al., 2011).

Sporadic outbreaks of severe pantropic enteritis, with severe clinical signs accompanied by a high mortality rate have been reported (Buonavoglia et al., 2006). These appear to be due to spontaneous emergence of virulent strains in susceptible young pups.

Previous serological studies indicate that infections with canine enteric coronaviruses are common (Tennant et al, 1991) and spread rapidly amongst susceptible dogs kept in close confinement in unhygienic conditions. Tennant et al. reported detection of antibodies in 54% of a population of healthy and diarrheic pet dogs in the United Kingdom, while CECoV seroprevalence ranged from 76% in a rescue kennel to 100% in a commercial breeding colony (Tennant et al., 1993). These studies demonstrate that seroprevalence rates depend on the population of dogs tested with generally higher rates in endemically infected kennels, where population densities are high and there is a continuous influx of susceptible animals and pathogens as a result of high dog turnover. In the United States, the seroprevalence of CECoV was 26% for privately owned pet dogs and up to 87% for kennelled dogs (Helfer-Baker et al., 1980). A CECoV prevalence of 2.8% was reported in a cross section of dogs presented to veterinary clinics in the UK when determined by RT-PCR from faecal samples (Stavisky et al, 2008). The Austrian partner of this project performed serological studies to estimate the occurrence and frequency of CECoV in the Austrian dog population (Spiss el al., 2012).

Their investigation revealed a seroprevalence of 88.2% whilst the virus prevalence detected in dogs with enteric disease was 31.3%.

4.2.2 CRCoV

CRCoV was isolated first in the United Kingdom from the nasal and pharyngeal swabs of dogs with acute respiratory signs (Erles et al., 2003). Tissue samples taken from the respiratory tract of diseased dogs were tested for the presence of coronaviruses using RT-PCR. Sequence analysis of four positive samples showed the presence of a coronavirus with high similarity to both BoCoV and HCoV-OC43 in their polymerase and S genes, whereas there was a low similarity to comparable genes in the enteric canine coronavirus. The virus was subsequently detected in several other countries in dogs also suffering from acute respiratory signs (Decaro et al., 2007, Kaneshima et al., 2006, Preistnall et al., 2006).

The pathogenesis of CRCoV is not entirely known yet but it is regarded as a pathogen in the poly-aetiological disease known as canine infectious respiratory disease complex (CIRD). Other CIRD pathogens include canine adenovirus 2 (CAD 2), canine parainfluenza virus (CPIV), canine influenza virus (CIV), canine herpes virus (CHV), *Streptococcus equi* subsp. *zooepidemicus*, *Bordatella bronchiseptica* and mycoplasma species (Erles et al., 2008). These pathogens can produce clinical signs, alone or in combination, that are virtually indistinguishable from one another. Diagnostic laboratory testing is required in order to identify the specific pathogens in the background. Typical clinical signs include coughing, nasal discharge and mild pyrexia. Occasionally more severe clinical signs develop with lower respiratory involvement and more destructive secondary bacterial infections of the respiratory tract (Quinn et al., 2011). Such cases can result in death if left untreated.

CIRD is usually only a problem when groups of dogs are kept together under crowded conditions, such as in animal shelters, laboratory animal units, and training kennels (Quinn et al., 2008). Despite widespread vaccination, CIRD remains a persistent global problem. In addition to the obvious welfare implications and costs of treatment, the disease also delays and disrupts re-homing and training schedules of kennels and shelters.

The seroprevalence of CRCoV in the domestic canine population has been shown to be 59.1% in Canada, 54.5% in the United States, 36.0% in the United Kingdom, 30.3% in the Republic of Ireland, and 17.8% in Japan (Kaneshima et al., 2006, Preistnall et al., 2006). The seroprevalence of CRCoV has been shown to increase with age in both UK and US canine populations and to decline following a plateau phase between 2 and 11 years (Preistnall et al.,

2006). The Austrian partner of this project performed serological studies to estimate the occurrence and frequency CRCoV in the Austrian dog population (Spiss el al., 2012). Their investigation revealed a seroprevalence of 61.2% in Austrian dogs and a virus prevalence of 8.8% detected in Austrian dogs with respiratory symptoms.

5. Materials and Methods

296 serum samples were taken at random from privately owned and clinically healthy Hungarian dogs. 57 serum samples were taken at random from dogs housed in three dog shelters in Hungary;

1. Shelter A, n=25: “Arvacskak” - Gyomro (total population ~ 190 dogs)
2. Shelter B, n=19: “Arvacska” - Szentendre (total population ~ 120 dogs)
3. Shelter C, n=13: HEROSZ - Godollo (total population ~ 50 dogs)

109 faecal samples were taken from Hungarian dogs showing clinical signs of enteritis; 81 from the Small Animal Clinic at Szent Istvan University in Budapest, 9 from private veterinary clinics, 5 from a dog breeder and 14 from four dog shelters. 94 intestinal and lung samples were taken from dogs dying due to symptoms of enteritis similar to a canine enteric coronavirus infection.

108 nasal/pharyngeal samples were taken from 108 Hungarian dogs with respiratory signs; 87 from 9 different dog shelters, 8 from a dog kennel and 13 from patients at the small animal Clinic at Szent Istvan University. 47 lung samples were obtained from the Institute of Pathology University of Veterinary Medicine, Vienna, taken from Austrian dogs that succumbed to respiratory disease characteristic of coronavirus infection.

Study 1: Serological survey of the prevalence of CECoV infection in Hungarian dogs.

Samples: Two cohorts of serum samples were collected for analysis. In the first cohort 278 serum samples were taken at random from privately owned and clinically healthy Hungarian dogs. In the second cohort 57 serum samples were taken at random from dogs housed in three dog shelters in Hungary;

1. Shelter A, n=25: “Arvacskak” - Gyomro (total population ~ 190 dogs)
2. Shelter B, n=19: “Arvacska” - Szentendre (total population ~ 120 dogs)
3. Shelter C, n=13: HEROSZ - Godollo (total population ~ 50 dogs)

Sample processing:

The investigations were performed at the laboratory in the Veterinary Diagnostic Directorate, National Food Chain Safety Agency, Budapest. The sera underwent virus neutralisation analysis following the standard protocol described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013 of the World Organisation for Animal Health (OIE). The test used was an adaptation of the virus neutralisation protocol set out for identification of transmissible gastroenteritis in pigs (http://www.oie.int/fileadmin/Home/eng/Health standards/tahm/2.08.11_TRANSMISSIBLEGASTRO.pdf). The prototype CECoV strain (Df-2) was propagated in a FCWF (felis cati whole foetus) permanent cell line. The test was validated by testing its reaction with positive reference sera. The test sera were two and three fold serially diluted in minimal essential medium (MEM) and 100 $TCID_{50}$/ml (tissue culture infectious dose) of virus was added. The samples were incubated for 1 hour at 37 °C and then inoculated on cell cultures. Cells were incubated at 37 °C, 5% CO_2 and were checked for cytopathic effects (CPE) on days 3-5 by light microscopy. Serum neutralisation titres were determined as the reciprocal of the serum dilution. The virus neutralisation test is one of the most specific methods of virus identification as it detects the anti viral antibody level in the serum however its sensitivity (titres) might be lower than other serological methods (i.e. the indirect IFA used in Study 3)

Study 2: Detection of CECoV in clinically ill and post mortem Hungarian dogs.

Samples:

A targeted survey was performed on the occurrence of CECoV in outbreaks of enteritis in Hungarian dog populations in Hungary between 2011 and 2013. 109 faecal samples, taken from dogs showing clinical signs of enteritis, were tested for the presence of CECoV at the Department of Pathology, Faculty of Veterinary Science, Szent Istvan University. The samples were obtained from the Small Animal Clinic at the Faculty of Veterinary Science, Szent Istvan University (n=81), a dog breeder in Budapest (n=5), private veterinary clinics in Budapest (n=9) and four dog shelters (n=14). Additionally, intestinal tissue samples were collected from 94 dogs that had died due to symptoms (enteritis and exsiccosis) characteristic of CECoV infections.

Sample processing:

Samples were homogenised and viral RNA was extracted using QIAamp® viral RNA mini kit (Qiagen, Carlsbad, USA). The extracted viral RNA was then analysed with RT-PCR assay as described by Decaro et al., 2005. The Qiagen one-step RT-PCR kit® was used for the amplification reactions. Samples were also tested with the SYBR Green real-time RT-PCR assay® for the detection of generic coronaviruses, described by Esutenaire et al., 2007. CECoV RNA positive extracts were subjected to nucleotide sequence determination at the institute of Virology, University of Veterinary Medicine, Vienna.

Nucleotide sequencing procedure:

For sequencing a region was chosen from the highly conserved 3'-untranslated region (3'-UTR) of the FeCoV genome (*Alphacoronavirus*) published by Herrewegh el al., 1995. This RT-PCR assay amplifies a 177 bp fragment (29-205 interval) using the following primers;

1. Forward primer: 5'- CCGAGGAATTACTGGTCATCGCG - 3'
2. Reverse primer: 5'- GCTCTTCCATTGTTGGCTCGTC - 3'.

Viral genome amplification was confirmed by direct sequencing of the amplified products in both directions (DNA Sequencing Service from Microsynth, Balgach). Sequences were compared on both sense and antisense strands for consensus, assembled, aligned and analysed using BioEdit Sequence Alignment® software. Consensus sequences were identified using the Basic Local Alignment Search Tool (BLAST) in gene bank databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Study 3: Serological survey of the prevalence of CRCoV infection in Hungarian dogs.

Samples:

The same sera used in study 1were also used in this study to investigate for the presence of CRCoV antibodies (*Betacoronavirus*); 278 samples extracted from privately owned dogs and 57 from three dog shelters.

Sample processing:

CRCoV antibodies were detected using indirect immune fluorescence assay (IFA). Madin Darby Bovine Kidney (MDBK) cells were cultivated on 96 well microtiter plates and after incubation overnight at 37°C in a humid 5% CO_2 atmosphere, inoculated with BoCoV strain 9/W/BL/77. After 1-2 days incubation the cells were washed three times with phosphate buffered saline (PBS) and fixed with 96% ethanol. Twofold dilutions of the dog sera, starting `with 1:10 were incubated (one well per dilution) for 30 minutes at 37°C. After three washes with PBS anti-dog-FITC (fluorescein isothiocyanate) was added to each well and incubated for 30 minutes at 37°C. After another three washes with PBS, counter staining with Eriochrome black for 5mins and another three PBS washing cycles the wells were evaluated using an inverse ultraviolet microscope. A cut-off value of 1:20 or more was regarded as a positive identification of CRCoV. The procedure was described in detail by Spiss et al., 2012. The investigations were performed at the Institute of Virology, University of Veterinary Medicine, Vienna.

Study4: Detection of CRCoV in clinically ill and post mortem Hungarian and Austrian dogs.

Samples:

108 Nasal/pharyngeal swab samples were collected from Hungarian dogs with respiratory signs; 87 samples from 9 dog shelters, 8 from a kennel and 13 from patients at the Small Animal Clinic at Szent Istvan University. The samples were tested for CRCoV at the Department of Pathology, Faculty of Veterinary Science, Szent Istvan University. Additionally, 47 lung samples of Austrian dogs, obtained from the Institute of Pathology, University of Veterinary Medicine, Vienna were also investigated for the presence of CRCoV specific nucleic acids.

Sample processing:

The samples were tested for CRCoV RNA using a SYBR Green real-time reverse RT-PCR assay for the presence of generic coronaviruses according to the method described by Escutenaire et al., 2007 and with the RT-PCR system described by Decaro et al., 2005.

From the Austrian samples, five sections of 5µm thickness were cut from paraffin blocks under RNAse-free conditions. Total RNA purification from the FFPE tissue sections was performed using the DNA/RNA FFPE Kit (Qiagen®). The paraffin was dissolved under

optimised lysis conditions and the nucleic acids were extracted according to the manufacturer's protocol. Realtime RT-PCR was performed using SuperScript III Platinum One-Step Quantitative RT-PCR System® (Invitrogen, Life Technologies, Carlsbad, USA) with primers and probes amplifying a region of the polymerase gene as described by Spiss et al., 2012.

6. Results

Study 1

The adapted virus neutralisation test performed on the serum samples detected CECoV neutralising antibodies in 76 of the 335 serum samples tested, giving an average seroprevalence of 22.7%. There was however a significant difference in prevalence amongst privately owned dogs and shelter dogs as illustrated in Table 1.The prevalence in shelter dogs was much higher at an average of 79.6% compared with the prevalence in privately owned dogs at 11.9%. There was also considerable variation amongst the seroprevalence of individual dog shelters varying from 60.0% seroprevalence in Shelter A up to 100.0% in Shelter C.

Table 1: Results of virus neutralisation tests for CECoV in Hungarian Dog sera.

Dogs	Number of sera	Positive sera	Seroprevalence (%)
Privately owned	278	33	11.9
Shelter A	25	15	60.0
Shelter B	19	15	78.9
Shelter C	13	13	100.0
Total	335	76	22.7

Serum antibody titres varied between 1:3 (cut-off) and 1:243. Details of the antibody titre values are shown in the appendix tables 1 and 2.

Study 2

CECoV RNA was detected in 28 of the 109 faecal samples investigated using the RT-PCR methods described by Decaro et al. 2005 and Escutenaire et al. 2007. Subsequently the CECoV RNA extracts were subjected to nucleotide sequence determination at the institute of Virology, University of Veterinary Medicine, Vienna. All samples were found to be positive for alphacoronavirus RNA fragments except for two where non-specific amplification products were detected (number 23 and 28 in table 2). Sequencing results for the RNA

fragments revealed 100% homogeneity of the analysed region between the samples. BLAST analysis of this fragment confirmed 100% homogeneity when compared to FeCoV (of the *Alphacoronavirus* genus), strain *Felis catus*/NLD/UU88/2010 (Genbank AccNo. KF530123). This analysis confirmed the presence of alphacoronavirus in 26 samples. The RT-PCR testing results of the samples and the sequencing results of the amplification products are shown in table 2.

Table 2: Results of RT-PCR of faecal samples and the sequencing results of the amplification products of 28 samples from Hungarian dogs

Case no.	PCR for CECoV	Sequencing for alphaCoV
1	Positive	Positive
2	Positive	Positive
3	Positive	Positive
4	Positive	Positive
5	Positive	Positive
6	Positive	Positive
7	Positive	Positive
8	Positive	Positive
9	Positive	Positive
10	Positive	Positive
11*	Positive	Positive
12	Positive	Positive
13	Positive	Positive
14	Positive	Positive
15	Positive	Positive
16	Positive	Positive
17	Positive	Positive
18	Positive	Positive
19*	Positive	Positive
20	Positive	Positive
21	Positive	Positive

22	Positive	Positive
23	Positive	Inconclusive
24	Positive	Positive
25	Positive	Positive
26	Positive	Positive
27	Positive	Positive
28	Positive	Inconclusive

* Dogs with respiratory signs (used in study 4).

Study 3

Indirect immunofluorescence assays performed on the serum samples to detect CRCoV revealed 149 positive samples of the 353 total giving an average seroprevalence of 42.7%. Similarly to study 1 this test revealed a considerable difference between the seroprevalence of CRCoV in privately owned Hungarian dogs (36.8%) compared with shelter dogs in Hungary (67.0%). There was also considerable variation amongst the seroprevalence of individual dog shelters varying from 42.2% seroprevalence in Shelter C up to 78.9% in Shelter B.

Table 3: Results of immunofluorescence assay for CRCoV in Hungarian dog sera.

Dogs	Number of sera	Positive sera	Seroprevalence (%)
Privately owned	296	109	36.8
Shelter A	25	19	76.0
Shelter B	19	15	78.9
Shelter C	13	6	46.2
Total	353	149	42.2

Serum antibody titres varied between 1:20 (cut-off) and 1:1280. Details of the antibody titre values are shown in the appendix tables 1 and 2.

Study 4

CRCoV RNA was not detected in any of the 108 nasal/pharyngeal Hungarian dog samples when tested with the RT-PCR methods described by Decaro et al., 2005 and the SYBR Green RT-PCR methods described by Eustenaire et al., 2007.

From the 47 lung samples from Austrian dogs, 6 samples revealed amplification products using RT-PCR for CRCoV RNA at the University of Veterinary Medicine in Vienna. Three of these samples were sequenced by Microsynth in Vienna, Austria. Two of the obtained sequences were suitable for further analysis and were sent for comparative sequence analysis with the polymerase gene sequences already existing in the GenBank archive for the coronavirus genome. The two samples confirmed 100% homology with CRCoV strain k37 (Genbank account number JX860640). The results are shown in table 4.

Table 4: CRCoV RT-PCR results on lung samples from Austrian dogs with respiratory disease.

Protocol Number	Age of the dog	CRCoV RT-PCR result	Remark
A834/04	8y	Negative	
C2229/04	8y	Negative	
C286/04	Unknown	Negative	
C827/05	Puppy, few weeks	Positive	Sequenced: CRCoV
D1426/05	1.5y	Negative	
D1850/05	11.5y	Negative	
D2080/02	4m	Negative	
D262/03	Puppy, few weeks	Positive	Sequenced: CRCoV
D410/04	2m	Weakly Positive	
D550/05	7y	Negative	
H1683/03	3m	Negative	
H248/02	Adult	Negative	
H539/02	3y	Negative	
T1408/05	2m	Negative	
T170/13	Unknown	Negative	
T195/13	Unknown	Weakly Positive	
T2482/03	3w	Negative	
T308/13	Unknown	Negative	
V1204/02	5.5y	Negative	
V163/03	6y	Negative	
V164/03	11y	Negative	CDV Negative
V1467/03	4m	Negative	CDV Positive
V665/03	4d	Negative	
W2222/02	8w	Negative	
W2223/02	8w	Negative	
W706/02	13y	Negative	CDV Negative
X1009/06	19d	Negative	

X1064/05	9.5y	Negative
X1104/07	3m	Negative
X1177/07	2w	Negative
X1534/05	8w	Negative
X1689/06	6.5y	Negative
X1851/06	10m	Negative
X2033/05	4.5y	Negative
X2039/06	3m	Negative
X273/07	2m	Negative
X384/04	3.5y	Negative
X384/05	8m	Negative
X44/06	4m	Negative
X569/07	6.5y	Negative
X633/05	3m	Weakly Positive
X778/02	Puppy, few weeks	Negative
X779/02	Puppy, few weeks	Negative
X92/07	Few Months	Negative
Y1907/02	11w	Negative
Y809/02	7d	Weakly Positive
X990/04	3w	Negative

7. Discussion

The serological results revealed an average of 22.7% seroprevalence of neutralising antibodies to CECoV in clinically healthy Hungarian dogs. Austria reported a seroprevalence of 69.9% in the clinically healthy Austrian dog population (Möstl et al., 1994) and a seroprevalence of as high as 88.2% in privately owned Austrian dogs with diarrheal disease and 0% in privately owned Austrian dogs without diarrheal disease (Spiss et al., 2012). It must be taken into consideration that the Austrian studies were carried out using IFA which is known to result in higher titres compared with the VN method used in this study. Seroprevalence clearly varies between countries and also the detection methods used. High seroprevalence of 91% are reported from Italy using an ELISA method for detecting antibodies in serum (Pratelli et al., 2002; Priestnall et al., 2007) where as much lower seroprevalence of 44% were reported in Japan using VN (Bandai et al., 1999). It is interesting to note that 17% of seropositivity was found in the sera used in this study that had been collected in 2006, while the seroprevalence was less than 10% in the sera collected between 2010 and 2012 (8.7% in 2010, 8.3% in 2011 and 9% in 2012).

Although the average seroprevalence in the Hungarian dog population was 22.7% there were significant differences in the seroprevalence between privately owned and shelter dogs. The seroprevalence in privately owned dogs was 11.8% with an average titre of 1:30.88 in the positive samples and a standard deviation of 47.6. In shelter A 60% of the dogs had VN antibodies with an average titre of 1:26.89 and standard deviation of 21.5. In shelter B there was a seroprevalence of 78.9% with an average titre of 1:26.1 and standard deviation of 24.19. In shelter C there was a seroprevalence of 100% with an average titre of 1:49.89 and standard deviation of 21.07. These results indicated a much higher number of dogs are infected with CECoV in shelter housing. The data also shows that 45% of the privately owned dogs had low antibody titres (below 1:15) compared with 13% in shelter A, 6% in shelter B and 0% in shelter C indicating higher levels of infection in seropositive dogs in sheltered housing compared to privately homed seropositive dogs.

These findings can be attributed to the fact that the two sample populations had very different epizootiological situations. Privately owned dogs have less frequent contact with other dogs

and generally come into contact with the same dogs. Therefore they are less likely to be exposed to CECoV. It must also be considered that the vaccination history of some of the privately owned dogs was not known which complicates the interpretation of the results as vaccinated dogs may be mistaken for infected dogs. In sheltered housing dogs are more frequently exposed to CECoV but the clinical manifestation of the disease was rare in the shelters involved in this study. Mild diarrhoea without mortality occurred in the winter - spring period only. At the time of sampling (July 2013) dogs were clinically healthy, well nourished and hygienic standards were fair. The dogs in shelter A were kept on sandy ground compared to the concrete ground found in shelters B and C. The 100% seropositivity revealed in shelter C may be attributed to the housing as the dogs in this shelter were kept in groups of 2-3 and indirect contact between all groups of dogs was frequent.

CECoV RNA was found in 25.7% of the faecal samples taken from diarrhoeic Hungarian dogs. This figure was reported as 31.3% in the equivalent study of the Austrian dog population (Spiss et al., 2012). The CECoV RNA extracts were subjected to nucleotide sequence determination and all samples were found to be positive for alphacoronavirus RNA fragments except for two where non-specific amplification products were detected (Table 2). Therefore the prevalence can be even lower (23.9%) among Hungarian dogs tested in this study, if we consider that the sequencing of the amplicon did not always prove the presence of coronavirus in the samples found positive by PCR. Several of the dogs in the study were immunised against canine distemper virus and 6 of the positive samples were taken from dogs co-infected by canine parvovirus type 2. A previous survey on the presence of CECoV in western European dogs with enteric disease reported varying prevalence between countries; 6% in Spain, 27.1% in the UK, 36.4% in Portugal, 43.4% in Italy, 55.5% in Greece and 78.1% in Hungary (Decaro et al., 2011). In this former survey the ratio of positive samples could be higher, because the collection of samples was much more targeted to enteritis outbreaks causing fatal cases than in the Austrian study, and samples were tested by PCR mostly in cases when histopathology findings supported the diagnosis of enteritis.

This study revealed an average CRCoV seroposativity of 42.2% in the Hungarian dog population using IFA methods. The seroprevalence in the privately owned dogs was lower (36.8%) than in the sheltered dogs (76% in shelter A, 78.9% in shelter B and 46.2% in shelter C). The seroprevalence in the privately owned Hungarian dog population was lower than those reported for the same study of the Austrian privately owned dogs (61.2%) (Spiss et al., 2012). When compared to other European countries similar seroprevalence figures are

reported in the UK (36.0%), the Republic of Ireland (30.3%) (Priestnall et al., 2006) and in Italy (32.06%) (Decaro et al., 2007) but higher seroprevalence is reported in United States (54.5%) and Canada (59.1%) (Priestnall et al., 2006).

The average CRCoV antibody titres in Hungarian dogs with private owners was 1:227 with a standard deviation of 242, in shelter A it was 1:231 with a standard deviation of 143, in shelter B 1:341 with a standard deviation of 133 and in shelter C it was 1:133 with a standard deviation of 97. The data shows a higher variance in titre volumes with the privately owned dogs which may be the consequence of the different vaccination backgrounds of these dogs as this information was unknown. The shelter with the highest seroprevalence of CRCoV, shelter B, also displayed the highest mean titre volumes and similarly the shelter with the lowest seroprevalence, shelter C, had the lowest mean titre volumes.

CRCoV RNA was not detected in any of the nasal and pharyngeal samples taken from Hungarian dogs, which may indicate the inadequacy of the primers applied in the tests. Further investigations on the same samples (stored at -86 ^{o}C) with a set of modified primers is planned. In the equivalent Austrian study of dogs with respiratory signs, 8.8% of the 34 samples tested positive for CRCoV specific nucleic acids. This correlates with the results from 47 lung samples used in this study which were taken from Austrian dogs that had died from respiratory disease. Of these samples, 12.8% tested positive for CRCoV specific nucleic acids.

The serological results for CRCoV infection in Hungarian dogs indicate that there is a higher prevalence of CRCoV infection in Austria compared with Hungary. It must be taken into consideration however that even between separate investigations in the same country the results can vary remarkably as illustrated in the UK where a 26.9% seroprevalence was reported in 2003 (Erles et al., 2003) and then a 0% seroprevalence was reported in 2005 (Erles and Brownlie, 2005). Regional and seasonal variations as well as population characteristics influence the results significantly.

8. Summary

The presence of CECoV and CRCoV was previously demonstrated in the Hungarian dog population by Lakatos et al in 2013. This study quantified the prevalence of these viruses in Hungarian dogs of different epizootiological conditions and different disease statuses. The study is part of a joint project with the University of Veterinary Medicine, Vienna who carried out a similar study on the Austrian dog population.

The prevalence of CECoV was investigated using two different methodologies. Firstly serum samples were taken from clinically healthy dogs, both privately owned (278 samples) and dogs housed in three different Hungarian shelters (57 samples). Virus neutralisation testing of the samples revealed 11.9% seropositivity in privately owned dogs and 79.6% seropositivity in sheltered dogs. Secondly faecal samples were taken from 109 diarrhoeic Hungarian dogs and tested for the presence of CECoV RNA using RT-PCR. The amplicons of the positive samples were subjected to nucleotide sequencing determination for alphacoronavirus RNA fragments. 25.7% of the samples revealed CECoV RNA following RT-PCR however two of these samples found positive by PCR revealed non-specific amplification products following nucleotide sequencing indicating that the true prevalence may be 23.9% among the dogs tested in the study.

The prevalence of CRCoV was also investigated using both serological and virological methods. Serological examinations using the same serum samples used in the CECoV investigation revealed a seropositivity of 36.8% in privately owned Hungarian dogs and 67% in sheltered Hungarian dogs using an indirect immunofluorescence detection technique. Virological examination of 108 nasal and pharyngeal swab samples taken from privately owned and sheltered Hungarian dogs with respiratory symptoms using RT-PCR for CRCoV RNA revealed no positive samples. The interpretation of this result should be guarded as it was most likely due to the inadequacy of the primers applied in the tests. Further investigations of the same samples (stored at -86 ^{o}C) with a set of modified primers is recommended. Subsequently 47 lung tissue samples obtained from the University of Veterinary Medicine Vienna were tested using an RT-PCR kit for CRCoV RNA that utili-sed different primers. 6 of these samples tested positive for CRCoV RNA (12.8% positivity).

9. Összefoglalás (Summary in Hungarian)

A kutyák kétféle coronavírus (CECoV és CRCoV) fertőzöttségét Lakatos és munkatársai (2013) már korábban kimutatták Magyarországon. A Bécsi Állatorvos-tudományi Egyetemmel közösen végzett vizsgálatunk ezeknek a vírusoknak a különböző járványtani feltételek között tartott kutyapopulácoókban való előfordulását mérte fel.

A CECoV előfordulását két módszerrel vizsgáltuk. Egyrészt szérummintákat gyűjtöttünk egészséges gazdás kutyákból (278 minta) és kutyamenhelyeken tartott állatokból (57 minta). A vírusneutralizációs próbában a gazdás kutyák 11,9%-a, a menhelyi kutyák 79,6%-a bizonyult pozitívnak. Másrészt bélsármintákat gyűjtöttünk 109 hasmenéses kutyából, melyeket RT-PCR módszerrel megvizsgáltunk a CECoV RNS jelenlétére. A pozitív minták amplikonjait szekvenáltattuk, hogy igazoljuk az alphacoronavírus jelenlétét. A minták 25,7%-a lett pozitív, de két mintában nem specifikus amplifikációs termék jelenlétét igazolta a vizsgálat, ezért valójában a prevalencia 23,9%-nak bizonyult.

A CRCoV jelenlétét ugyancsak két módszerre, szerológiai és víruskimutatási eljárással is vizsgáltuk. Az indirect immunfluoreszcenciás vizsgálat 36,8% pozitivitást mutatott ki a gazdás kutyák és 67% pozitivitást a menhelyi kutyák körében. A 108 orr- és garattampon közül egy sem bizonyult pozitívnak a magyarországi gazdás és menhelyi kutyákból vett minták esetében. Ezt az eredményt óvatosan kell kezelni, mert nagy valószínűséggel az alkalmazott primerek nem voltak megfelelőek. A -80 ^{o}C-on tárolt minták újabb vizsgálata szükséges, módosított primerek felhasználásával. A Bécsi Állatorvos-tudományi Egyetemen gyűjtött 47 tüdőminta RT-PCR vizsgálatával hatot találtunk pozitívnak CRCoV RNS jelenlétére, ez 12,8% pozitivitást jelent.

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12. Appendices

Appendix Table 1:Detection of anti-CECoV antibodies with virus neutralisation (VN) test and anti-CRCoV antibodies with immunofluorescence assay (IFA) in sera of Hungarian dogs with known owners.

Nr.	Sample ID	Sampling date	Date of birth	CECoV VN titre	CRCoV IFAtitre
1	34192	20.12.2012	01.05.2004	toxic	negative
2	33401	14.12.2012	03.03.2012	toxic	negative
3	33536/1	17.12.2012	15.08.2012	negative	negative
4	34236	21.12.2012	01.04.2000	negative	1:80
5	32490	10.12.2012	19.11.2009	negative	1:160
6	29259/3	13.11.2012	02.08.2012	negative	negative
7	29525	15.11.2012	07.07.2012	negative	negative
8	28570	07.11.2012		negative	1:640
9	28574	07.11.2012	01.06.2012	toxic	1:1280
10	28599	07.11.2012	05.06.2012	toxic	negative
11	19128	20.07.2012	06.06.2010	negative	1:320
12	15068	15.06.2012		negative	negative
13	13322	31.05.2012	04.04.2004	negative	negative
14	28602	07.11.2012		negative	negative
15	33571	17.12.2012	23.07.2009	negative	1:320
16	365	07.01.2013	21.01.2006	1:9	1:40
17	6929	28.03.2012		negative	negative
18	6382	23.03.2012	10.11.2011	1:5.2	1:320
19	31227	29.11.2012		negative	1:20
20	30501	23.11.2012	08.06.2012	negative	negative
21	32059	05.12.2012	28.09.2002	1:5.2	1:320
22	7624			negative	negative
23	4550	02.03.2012		negative	negative
24	4746			negative	negative
25	24539	27.09.2012	01.05. 2012	negative	negative
26	25067	03.10.2012		1:46.8	1:320
27	25098	03.10.2012		negative	negative
28	31644	01.12.2012	24.04.2012	1:3	negative
29	118	03.01.2013		negative	negative
30	22338	04.09.2012	19.02.2007	negative	1:320
31	22562	06.09.2012	20. 07.2002	negative	1:160
32	11618	15.05.2012		negative	1:320
33	10789	08.05.2012		negative	1:80
34	11031	09.05.2012		negative	negative
35	22384	04.09.2012	19.03.2012	negative	negative
36	9421/1	24.04.2012		negative	negative
37	9421/2	24.04.2012		negative	negative

38	8687	18.04.2012		negative	1:320
39	9817	26.04.2012		negative	negative
40	6613	26.03.2012	22.03.2011	toxic	negative
41	12001	17.05.2012		negative	1:20
42	4287	28.02.2012		negative	negative
43	4404	29.02.2012		negative	negative
44	5034	07.03.2012		negative	1:20
45	5145	08.03.2012		negative	negative
46	27572	29.10.2012	26.06.2010	1:46.8	1:640
47	27996	31.10.2012		negative	negative
48	23498	18.09.2012		negative	1:160
49	27929	24.10.2011	10.06.2011	negative	negative
50	27924	24.10.2011		negative	1:20
51	27815	21.10.2011	17.01.2011	negative	negative
52				toxic	1:40
53	32813	08.12.2011		negative	1:20
54				toxic	1:20
55	32099	01.12.2011		negative	negative
56	34399	27.12.2011		negative	negative
57	1183	17.01.2012		negative	negative
58	1458/1	20.01.2012		negative	negative
59	1458/2	20.01.2012		negative	negative
60	5067/1	08.03.2012		negative	negative
61	21145/2	16.08.2012		negative	negative
62	29882	14.11.2011		negative	1:40
63	29069/1	07.11.2011		negative	1:640
64	29069/3	07.11.2011		negative	negative
65	27921/3	24.10.2011		negative	negative
66	28434	28.10.2011		negative	negative
67	27518	20.10.2011		negative	negative
68	33181	14.12.2011	01.09.2010	negative	1:40
69	32253	02.12.2011		negative	negative
70	33026	12.12.2011		negative	negative
71	33991	21.12.2011	20.07. 2003	1:3	negative
72	33993	21.12.2011		negative	negative
73	31641	29.11.2011		negative	negative
74	14064	07.06.2012	08.12.2011	negative	negative
75	16931	29.06.2012		negative	1:320
76	16831/2	28.06.2012		negative	negative
77	16831/1	28.06.2012		negative	1:320
78	15276	18.06.2012		negative	1:40
79	14383	11.06.2012		negative	1:20
80	14394	11.06.2012		negative	1:640
81	14076	07.06.2012		negative	1:80
82	14072	07.06.2012		negative	1:320
83	12709	24.05.2012		negative	1:80
84	14682/2	13.06.2012		negative	negative
85	14682/4	13.06.2012		negative	negative

86	14682/3	13.06.2012		negative	negative
87	12702	24.05.2012	15.03.2004	negative	1:40
88	12705	24.05.2012	2000	negative	1:160
89	21981	29.08.2012		negative	1:20
90	12665	24.05.2012		negative	negative
91	7708	05.04.2012		negative	1:20
92	7464	03.04.2012	24.08.2010	1:46.8	1:320
93	20303	06.08.2012		negative	negative
94	17953	06.07.2012		negative	1:160
95	17320	03.07.2012	21.05.2011	negative	negative
96	18213	10.07.2012		negative	negative
97	20464	07.08.2012		negative	negative
98	22021	30.08.2012		negative	1:640
99	21516/1	22.08.2012		negative	negative
100	20211	03.08.2012		negative	negative
101	2011-155	03.01.2011		negative	1:80
102	2010-34059	20.12.2010	17.06.2010	negative	negative
103	2010-34033	20.12.2010	06.08.2009	negative	negative
104	2010-32000	02.12.2010	11.09.2009	negative	negative
105	2010-32022 / 1	02.12.2010	08.07.2010	negative	negative
106	2010-33196	13.12.2010	17.08.2009	negative	negative
107	2010-31788	01.12.2010	02.08.2010	negative	negative
108	2010-28503	08.11.2010	25.06.2010	negative	negative
109	2010-28430	05.11.2010	16.06.2006	negative	1:320
110	2011-25511	28.09.2011	31.05.2011	negative	negative
111	2011-19477 / 2	08.07.2011	07.10.2006	negative	1:160
112	2011-19618	11.07.2011	28.12.2008	negative	negative
113	2011-18537	29.06.2011		negative	negative
114	2011-18483 / 1	29.06.2011		negative	negative
115	2011-18483 / 2	29.06.2011		negative	negative
116	2011-18483 / 3	29.06.2011		negative	negative
117	2011-18477 / 1	29.06.2011	04.10.2010	negative	negative
118	2011-18470	29.06.2011	18.10.2004	negative	1:80
119	2011-19477 / 1	08.07.2011	03.03.2004	negative	1:160
120	2011-19462	07.07.2011	31.05.2009	negative	negative
121	2011-19084 / 1	05.07.2011	04.08.2002	negative	1:160
122	2011-19121 / 1	06.07.2011	07.07.2003	negative	negative
123	2011-19121 / 2	06.07.2011	23.04.2010	negative	negative
124	2011-19121 / 3	06.07.2011		negative	negative
125	2011-19088	05.07.2011	11.06.2010	negative	negative
126	2011-19118	06.07.2011	03.04.2008	negative	1:320
127	2011-18635	30.06.2011	10.06.2007	negative	1:320
128	2011-18766	04.07.2011	20.05.2010	negative	negative
129	2011-25921	04.10.2011	05.06.2011	negative	negative
130	2011-26379	07.10.2011	18.03.2011	negative	negative
131	2011-26237	06.10.2011	16.06.2010	negative	negative
132	2011-22070 / 2	12.08.2011	01.01.2008	negative	negative
133	2011-22831 / 4	25.08.2011		negative	negative

134	2011-22394	17.08.2011	2007	1:46.8	1:320
135	2011-23080	30.08.2011	13.01.2004	1:81	1:160
136	2011-22510	18.08.2011	12.03.2007	negative	1:20
137	2011-22418	18.08.2011	24.09.2009	negative	1:320
138	2011-23210	31.08.2011	15.05.2007	negative	1:160
139	2011-22817	25.08.2011	20.09.2000	1:9	1:80
140	2011-23343	01.09.2011	14.09.2007	negative	1:80
141	2011-9868	06.04.2011		negative	negative
142	2011-22831	25.08.2011		negative	negative
143	2011-22831	25.08.2011		negative	negative
144	2011-9742	06.04.2011		negative	negative
145	2011-9811	06.04.2011		negative	negative
146	2011-9081	31.03.2011	30.05.2010	negative	negative
147	2011-24062	09.09.2011	02.10.2010	negative	negative
148	2011-23719 / 3	06.09.2011	09.05.2011	negative	negative
149	2011-23762 / 1	07.09.2011	09.05.2011	1:27	negative
150	2011-21150	01.08.2011	31.03.2011	negative	negative
151	2011-10728	13.04.2011	19.11.2010	negative	1:20
152	2011-10490 / 2	12.04.2011	13.12.2010	negative	negative
153	2011-10793 / 2	14.04.2011	14.12.2010	negative	negative
154	2011-10490 / 6	12.04.2011	08.12.2010	negative	negative
155	2011-10490 / 1	12.04.2011	08.12.2010	1:140.3	negative
156	2011-9523	05.04.2011		negative	negative
157	2011-24062 / 2	09.09.2011	20.03.2011	negative	negative
158	2011-24304 / 1	14.09.2011	17.03.2011	negative	negative
159	2011-24304 / 2	14.09.2011	17.03.2011	negative	negative
160	2011-22898	25.08.2011	23.11.2008	negative	negative
161	2011-26204 / 2	06.10.2011	01.01.2011	toxic	negative
162	2010-25362 / 1	13.10.2010	22.04.2008	negative	negative
163	2010-23716	29.09.2012	01.08.2007	1:15.6	negative
164	2010-23719	29.09.2010	25.09.2009	1:15.6	negative
165	2010-26162	19.10.2010	12.08.2007	negative	1:40
166	2010-24353	05.10.2010	09.2008	negative	negative
167	2010-25035	11.10.2010	01.042000.	negative	1:320
168	2010-21613	07.09.2010		negative	1:160
169	2010-21974	10.09.2010	17.04.2008	1:27	1:160
170	2010-25432	13.10.2010	20.05.2007	negative	negative
171	2010-21613	07.09.2010		negative	1:160
172	2010-21648 / 1	08.09.2010	17.03.2009	negative	negative
173	2010-21648 / 2	08.09.2010	17.03.2009	negative	negative
174	2010-6752	25.03.2010		negative	negative
175	2010-27898	02.11.2010	30.062008.	1:9	1:160
176	2010-27883 / 1	02.11.2010	05.12.2007	1:243	negative
177	2010-27883 / 2	02.11.2010	18.10.2007	negative	negative
178	2010-28036	03.11.2010	01.01.2009	negative	1:320
179	2010-25758	15.10.2010	26.12.2006	negative	negative
180	2010-25918	18.10.2010		negative	1:80
181	2010-26833	25.10.2010	31.05.2010	negative	1:20

182	2010-10677	07.05.2010		negative	1:320
183	2010-10055	03.05.2010		negative	negative
184	2010-6861	26.03.2010		negative	1:20
185	2010-20322	19.08.2010	27.01.2010	negative	1:160
186	2010-20770	27.08.2010	12.02.2009	negative	negative
187	2010-26709	22.10.2010	16.04.2010	negative	negative
188	2010-18636	29.07.2010	20.06.2005	negative	negative
189	2010-19908 / 3			negative	1:320
190	2010-20114	18.08.2010	20.06.2005	negative	negative
191	2010-19817	13.08.2010		negative	negative
192	2010-18661	29.07.2010	05.04.2010	negative	negative
193	2010-18766	30.07.2010		negative	1:160
194	2010-21143	01.09.2010	18.05.2010	1:15.6	negative
195	2010-17537	16.07.2010		negative	negative
196	2010-18015	21.07.2010		negative	negative
197	2010-19504	10.08.2010		negative	1:40
198	2010-19314	06.08.2010		negative	negative
199	2010-18011	21.07.2010		negative	1:40
200	2010-19501	10.08.2010		negative	negative
201	41340/2009	28.12.2009		negative	1:160
202	41339	28.12.2009	11.10.2004	1:40	1:320
203	2037	13.01.2009	24.03.2006	negative	negative
204	38554	30.11.2009	06.01.2009	negative	negative
205	38815	02.12.2009	04.10.2006	negative	1:40
206	39008	02.12.2009	25.07.2008	negative	1:20
207	812/2010	13.01.2010	20.11.2005	negative	1:40
208	952/1	14.01.2010		negative	negative
209	952/2	14.01.2010		negative	negative
210	2284	28.01.2010		negative	negative
211	2385/1	29.01.2010	16.07.2009	toxic	negative
212	2385/2	29.01.2010	16.07.2009	toxic	negative
213	2496	01.02.2010	14.06.2007	negative	1:320
214	2596	02.02.2010		negative	negative
215	2241	28.01.2010	03.09.2009	negative	1:320
216	1060	14.01.2010		negative	negative
217	2719	03.02.2010		negative	negative
218	14925/2006	08.06.2006	02.09.2005	1:20	negative
219	14962	08.06.2006	11.05.2004	negative	negative
220	4042	10.02.2006		negative	negative
221	4041	10.02.2006	13.10.2005	negative	1:160
222	4043	10.02.2006	15.08.2004	negative	1:40
223	4702	17.02.2006	28.06.1999	negative	1:160
224	6808	06.03.2006	02.11.2003	negative	1:640
225	6776	06.03.2006	27.05.2005	1:10	1:80
226	7250	09.03.2006		negative	negative
227	7237	09.03.2006	01.08.2005	toxic	negative
228	6544	03.03.2006	31.05.2005	negative	1:320
229	6447	02.03.2006	01.02.2004	negative	1:640

230	6175	07.03.2011		negative	negative
231	6036	27.02.2006		negative	1:320
232	6445	02.03.2006	17.12.2004	negative	1:160
233	21614	25.08.2006	17.07.2005	negative	negative
234	21681	28.08.2006		negative	negative
235	18197	12.07.2006	12.03.2005	negative	negative
236	17622	06.07.2006	14.11.2004	negative	negative
237	18296	13.07.2006	29.09.2005	negative	negative
238	11919	03.05.2006		negative	negative
239	18441	14.07.2006		negative	negative
240	20071	04.08.2006	15.07.2001	1:14.5	1:20
241	20072	04.08.2006	14.07.2004	toxic	negative
242	22687/2	08.09.2006		negative	negative
243	22756	11.09.2006	13.10.2005	negative	negative
244	22804	11.09.2006	10.09.2003	negative	negative
245	22843	11.09.2006	03.01.2004	negative	1:80
246	21553/1	25.08.2006	19.01.2006	negative	negative
247	21553/2	25.08.2006	19.01.2006	negative	negative
248	21553/3	25.08.2006	19.01.2006	negative	negative
249	20303	08.08.2006	01.2005	negative	negative
250	21439	24.08.2006	02.08.2004	negative	negative
251	20597	11.08.2006	08.01.2006	negative	negative
252	21090	18.08.2006		negative	1:320
253	16875	28.06.2006		negative	negative
254	22507	07.09.2006	08.04.2005	negative	negative
255	16549	26.06.2006	16549	toxic	negative
256	18243	13.07.2006	28.10.2003	1:10	1:80
257	26238	19.10.2006	09.04.2006	toxic	negative
258	6300	01.03.2006		negative	1:320
259	6301	01.03.2006		negative	1:160
260	430	06.01.2006	13.12.2004	toxic	negative
261	2941	01.02.2006	17.11.2004	negative	negative
262	2274	25.01.2006		negative	negative
263	6297	01.03.2006		negative	1:160
264	6294	01.03.2006		negative	negative
265	6293	01.03.2006		negative	1:160
266	2995	01.02.2006	12.04.2005	1:10	1:80
267	1227	16.01.2006		1:14.5	1:320
268	14326/2	30.05.2006		negative	negative
269	14326/3	30.05.2006		negative	negative
270	14327	30.05.2006		negative	negative
271	14347	30.05.2006		negative	negative
272	13754	23.05.2006	03.11.2004	1:10	1:320
273	13888	24.05.2006	22.12.2005	toxic	negative
274	13163	16.05.2006		1:20	negative
275	12904	12.05.2006	31.10.2004	negative	1:320
276	13161	16.05.2006		1:10	negative
277	12844	11.05.2006		negative	negative

278	13966	25.05.2006		negative	negative
279	12562	09.05.2006		negative	1:320
280	12782	11.05.2006	13.06.1999	1:20	1:20
231	12867	12.05.2006	21.08.2005	negative	negative
282	9708	05.04.2006	06.08.2004	1:20	negative
283	9371	31.03.2006	1998	negative	1:320
284	12300	05.05.2006		negative	negative
285	11366	25.04.2006		toxic	negative
286	11495	26.04.2006	12.06.1995	negative	negative
287	10903	20.04.2006	25.09.2004	negative	1:20
288	10412	12.04.2006	12.01.2004	toxic	negative
289	10275	11.04.2006	10.10.2005	negative	negative
290	10414	12.04.2006	09.05.2005	1:14.5	negative
291	10901	20.04.2006	01.08.2005	1:10	negative
292	19747	01.08.2006	19.07.2005	negative	negative
293	20917	16.08.2006	18.04.2005	negative	negative
294	20990	17.08.2006	29.09.2003	negative	1:1280
295	28100	30.12.2005		negative	1:1280
296	28036	03.11.2010	01.01.2009	negative	1:160

CECoV VN cut-off: 1:3 serum dilution; CRCoVIFA cut-off: 1:20 serum dilution

Appendix Table 2:Detection of anti-CECoV antibodies with virus neutralisation (VN) test and anti-CRCoV antibodies with immunofluorescence assay (IFA) in sera of Hungarian dogs kept in shelters.

Nr.	**Sampling date**	**Name**	**CECoV virus neutralisation titre**	**CRCoV IFA titre**
A1	11.07.2013	no name	negative	1:80
A2	11.07.2013	no name	1:15.6	negative
A3	11.07.2013	no name	negative	1:160
A4	11.07.2013	no name	1:15.6	1:80
A5	11.07.2013	no name	1:46.8	negative
A6	11.07.2013	no name	1:5.2	1:80
A7	11.07.2013	no name	negative	1:320
A8	11.07.2013	no name	1:46.8	1:80
A9	11.07.2013	no name	negative	1:320
A10	11.07.2013	no name	1:15.6	negative
A11	11.07.2013	no name	negative	1:320
A12	11.07.2013	no name	negative	negative
A13	11.07.2013	no name	>1:81	negative
A14	11.07.2013	no name	1:15.6	1:160
A15	11.07.2013	no name	negative	1:320
A16	11.07.2013	no name	negative	1:640
A17	11.07.2013	no name	1:15.6	1:160
A18	11.07.2013	no name	negative	1:80

A19	11.07.2013	no name	1:15.6	1:320
A20	11.07.2013	no name	1:46.8	1:320
A21	11.07.2013	no name	negative	1:320
A22	11.07.2013	no name	1:46.8	1:320
A23	11.07.2013	no name	1:5.2	1:160
A24	11.07.2013	no name	1:15.6	negative
A25	11.07.2013	no name	1:15.6	1:160
B1	21.07.2013	Dorka	1:46.8	1:320
B2	21.07.2013	Negro	negative	negative
B3	21.07.2013	Mimi	1:46.8	1:320
B4	21.07.2013	Fiona	1:15.6	1:320
B5	21.07.2013	Masló	negative	negative
B6	21.07.2013	Don	1:46.8	1:160
B7	21.07.2013	Vis	1:46.8	1:320
B8	21.07.2013	Fruzsi	>1:81	1:320
B9	21.07.2013	Kara	1:15.6	1:320
B10	21.07.2013	Jenny	1:5.2	1:320
B11	21.07.2013	Győző	1:15.6	1:320
B12	21.07.2013	Marcipan	1:15.6	1:320
B13	21.07.2013	Luke	1:15.6	1:160
B14	21.07.2013	Rico	>1:81	1:640
B15	21.07.2013	Finci	1:15.6	1:320
B16	21.07.2013	Bundi	negative	negative
B17	21.07.2013	Lujzi	1:46.8	1:640
B18	21.07.2013	Zsebi	negative	negative
B19	21.07.2013	Opál	1:46.8	1:320
C1	28.07.2013	Csikós	1:46.8	1:80
C2	28.07.2013	P3	1:46.8	negative
C3	28.07.2013	Jenő	1:46.8	1:160
C4	28.07.2013	Dongó	1:15.6	negative
C5	28.07.2013	Janó	1:46.8	1:80
C6	28.07.2013	P1	1:46.8	negative
C7	28.07.2013	Csipesz	1:46.8	1:80
C8	28.07.2013	Bogáncs	1:46.8	1:80
C9	28.07.2013	Baha	>1:81	negative
C10	28.07.2013	P2	1:15.6	negative
C11	28.07.2013	Kifli	1:46.8	1:320
C12	28.07.2013	Alf	>1:81	negative
C13	28.07.2013	Alfa	>1:81	negative

CECoV VN cut-off: 1:3 serum dilution; CRCoV IFA cut-off: 1:20 serum dilution