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## Prevalence of feline coronavirus types I and II in cats with histopathologically verified feline infectious peritonitis

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### Abstract

Feline coronaviruses (FCoV) vary widely in virulence causing a spectrum of clinical manifestations reaching from subclinical course to fatal feline infectious peritonitis (FIP). Independent of virulence variations they are separated into two different types, type I, the original FCoV, and type II, which is closely related to canine coronavirus (CCV). The prevalence of FCoV types in Austrian cat populations without FIP has been surveyed recently indicating that type I infections predominate. The distribution of FCoV types in cats, which had succumbed to FIP, however, was fairly unknown. PCR assays have been developed amplifying parts of the spike protein gene. Type-specific primer pairs were designed, generating PCR products of different sizes. A total of 94 organ pools of cats with histopathologically verified FIP was tested. A clear differentiation was achieved in 74 cats, 86% of them were type I positive, 7% type II positive, and 7% were positive for both types. These findings demonstrate that in FIP cases FCoV type I predominates, too, nonetheless, in 14% of the cases FCoV type II was detected, suggesting its causative involvement in cases of FIP.

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### 1. Introduction

Feline infectious peritonitis (FIP) is a fatal, immune-mediated disease of domestic and wild fe-

lidae. The causative agent, the feline coronavirus (FCoV), is a member of the family Coronaviridae. Coronaviruses are divided into five antigenic groups. Group I includes FCoV, transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), canine coronavirus (CCV) and human coronavirus 229E (HCoV-229E) (Wege et al., 1982). Cats are susceptible to all group I coronaviruses (Horzinek

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et al., 1982; Wege et al., 1982). Barlough et al. (1985) showed that an infection with CCV caused seroconversion but no clinical signs in the cats examined, neither was the course of the subsequently experimentally induced FIP disease more severe. In contrast to these findings, McArdle et al. (1992) demonstrated that after infection with CCV the course of FIP disease was more severe, and that CCV induced in some cats similar symptoms as in the dog. Furthermore, one CCV strain caused in a cat clinical symptoms which were not discernible from FIP. After all, the importance of CCV for the cat remains uncertain (Sparkes et al., 1992).

Two biological types of FCoV are known, feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV) (Pedersen, 1976b, 1983, 1987; Pedersen et al., 1981). The genome of some FECV strains proved to be 0.3 kb shorter, suggesting a deletion of 300 bp at the 3'-end (Vennema et al., 1992). Molecular studies showed that FIPV arises by mutation from FECV (Pedersen et al., 1981; Evermann et al., 1991; Hök, 1993; Poland et al., 1996; Herrewegh et al., 1997; Vennema et al., 1994, 1998). Both FIPV and FECV may, depending on their virulence, cause viremia (Herrewegh et al., 1995; Fehr et al., 1996; Gunn-Moore et al., 1998; Horzinek, 2000).

FCoVs are separated into two different types based upon their growth ability *in vitro*, their antigenic relationship to CCV, their neutralisation reactivity with S-protein-specific mAbs (Fiscus and Teramoto, 1987a,b; Hohdatsu et al., 1991, 1992) and upon sequence analysis of the S-protein gene (Motokawa et al., 1995).

While type I shows no or little replication in cell culture (FIPV UCD1, UCD2, UCD3, UCD4, TN-406, NW1, Yayoi, KU-2, Dahlberg, FECV UCD), type II induces a lytic cytopathic effect (FIPV 79-1146, NOR15 (DF2), Cornell-1, FECV 79-1683). The ability of an FCoV strain to propagate in cell culture does not correlate with its virulence *in vivo* (Mochizuki et al., 1997). Among the FCoV types I and II, both FIPV and FECV strains are represented. The S-protein gene of type II is closely related to those of TGEV and CCV, showing a similarity of the nucleotide sequence of 91 and 81%, respectively, but of only 46% with the S-protein gene of type I (Motokawa et al., 1995). Herrewegh et al. (1998) demonstrated that FCoV type II resulted from recombination of FCoV type I with CCV.

Recent studies indicate that type II uses the feline aminopeptidase N (fAPN), a cell-surface metalloprotease on the intestinal, lung and kidney epithelial cells, as receptor, and that fAPN may also bind CCV, TGEV and HCV. It is not clear whether or not this receptor specificity of type II plays a role in the pathogenesis or pathological alterations of FIP (Williams et al., 1991; De Groot and Horzinek, 1995; Hohdatsu et al., 1998; Tresnan and Holmes, 1998).

The prevalence of types I and II has been surveyed in two studies from Austria and Japan, respectively, both suggesting that the majority of FCoV infections is due to type I (Hohdatsu et al., 1992; Posch et al., 1999, 2001). FCoVs are ubiquitous in the cat population, highly infectious by the oronasal route and therefore endemic in multi-cat households, catteries and shelters. Investigations showed that a high percentage of cats without FIP symptoms from exposed environments were positive for FCoV infection: 39–85% were seropositive, 37–95% viremic and 73–81% excreted virus in their faeces (Addie and Jarrett, 1992b; Sparkes et al., 1992; Herrewegh et al., 1995; Foley et al., 1997a,b; Gunn-Moore et al., 1998). Posch et al. (1999, 2001) found 71% seropositive cats in Austrian cat populations without signs of FIP, 26% of these cats tested positive for FCoV nucleic acid in blood.

There is strong evidence for the existence of persisting and chronic infections, with virus persisting in the intestine and other organs of healthy cats. Asymptomatic carriers may excrete virus over a period of months or even years (Foley et al., 1997a; Herrewegh et al., 1995, 1997). Asymptomatic carriers and shedders represent coronavirus reservoirs and therefore the main problem in the prevention of FIP in multi-cat environments (Addie and Jarrett, 1992a,b; Addie et al., 1995, 1996; Foley et al., 1997a,b; Herrewegh et al., 1997).

Approximately 5–10% of seropositive cats develop FIP, with the highest incidence in cats between 6 months and 5 years of age, and the majority of cases occurring in cats  $\leq 1$  year of age (Scott, 1991; Addie and Jarrett, 1992a).

The higher incidence of FIP among purebred cats (Scott, 1991), cheetahs (Evermann et al., 1988) and cats from FIP-susceptible bloodlines (Foley and Pedersen, 1996) may be an indication for a genetic predisposition.

In addition, the sex of the host may influence the outbreak of the disease. While Pedersen (1976a) found no generic disposition, Potkay et al. (1974) and Binder and Hartmann (2000) observed a higher incidence of FIP among males than among females.

Although serological testing by immunofluorescence assay (IFA) (Moestl, 1983) or ELISA (Mochizuki and Furukawa, 1989) is a helpful tool for FIP diagnosis, results can only be interpreted in correlation with clinical symptoms (Sparkes et al., 1991). At present, the only conclusive FIP diagnosis can be established by histopathological examination of a biopsy or post mortem material.

The recently developed reverse transcriptase polymerase chain reaction (RT-PCR) assays, using primers targeted to highly conserved regions of the viral genome (3'-UTR (untranslated region) (Herrewegh et al., 1995; Fehr et al., 1996), or S-protein gene (Li and Scott, 1994; Gamble et al., 1997)), which are common to all FCoV strains, became a valuable tool for the detection of FCoV nucleic acid in blood, body cavity effusions, faeces and tissue samples of infected cats.

In particular the N-terminal domain of the S-protein gene allows a differentiation between the two types I and II. Posch et al. (1999, 2001) developed an RT-PCR using primers targeted to the S-protein gene to study the prevalence of the two FCoV types in cats without FIP symptoms, and showed that 55% of the PCR-positive cats proved positive for type I, 28% for type II and 17% for both types.

With the retrospective study presented here we investigated the prevalence of the two types of FCoVs in cats with histopathologically verified FIP using nested and seminested RT-PCR assays, with primers targeted as well to the S-protein gene. The aim of this study was to investigate the distribution of the two FCoV types in FIP diseased cats. Furthermore—since FCoV types I and II may use different receptors—we wanted to investigate whether the two types are associated with differences in the clinical course of the disease and/or distinct histopathological changes. Finally we intended to get more information about the importance of CCV for the cat. CCV itself may infect the cat, or it may be involved indirectly, regarding the possibility that recombinations between FCoV type I and CCV may happen in the field at any time (Horzinek, 2000).

Table 1

Breed, gender and age of the investigated cats with and without FIP-symptoms

	Percent cats without FIP ( <i>n</i> = 1600)	Percent cats with FIP ( <i>n</i> = 154)
Domestic cat	86.5	66.4
Purebred	13.5	<b>33.6</b>
Male	53.4	<b>62.4</b>
Female	46.6	<b>37.6</b>
[0–1] year	33.1	<b>52.1</b>
[1–2] years	8.2	14.3
[2–5] years	14.4	12.1
[5–8] years	12.9	9.3
>8 years	31.4	12.2

Statistically significant differences compared to cats without FIP are highlighted in bold.

## 2. Materials and methods

### 2.1. Breed, gender and age of the cats with FIP

Between 1997 and 2000 a total of 1754 cats were examined at the Institute of Pathology and Forensic Veterinary Medicine of the University of Veterinary Medicine, Vienna, and 154 of these cats were diagnosed with FIP. The analysis of breed, gender and age of the 154 cats with FIP compared to the 1600 cats without FIP symptoms is shown in Table 1.

The statistical evaluation of the parameters breed, gender and age in the two groups “cats with FIP” and “cats without FIP” was carried out by  $\chi^2$ -test using the program SPSS for MS Windows, version 8.0.

### 2.2. Tissue samples

From 94 of the 154 cats with histopathologically confirmed FIP organ samples (lung, liver, spleen, kidney, gut) were available, either formalin-fixed paraffin-embedded tissues (PET) (*n* = 65, 1997–1998) or fresh organ samples (*n* = 29, 1999–2000). PET samples had been fixed in buffered formaldehyde for 48 h and were then embedded in paraffin. Fresh organ samples were taken during section and either processed immediately or stored at  $-80^\circ\text{C}$  until used.

### 2.3. Preparation of paraffin-embedded tissues

The preparation of PET samples was carried out essentially as described by Sorg and Metzler (1995):

four to six 5 µm thick sections of paraffin-embedded organs from each cat were pooled and deparaffinised by incubating for 30 min in Xylene and washing twice for 5 min in ethanol at room temperature. After centrifugation and air drying for 10 min at 37 °C, 25–50 µl proteinase K and 200–400 µl (depending on the sample size) buffer ATL (Qiagen, Valencia, CA, USA) were added and the samples were then incubated at 37 °C for 5 days. When necessary, another equivalent of proteinase K and buffer ATL was added at the second or third day of incubation.

After inactivation of proteinase K at 95 °C for 8 min and centrifugation, RNA was extracted from the upper aqueous phase using a commercially available Kit (QIAamp Viral RNA Mini Kit, Qiagen, Valencia, CA, USA). The extracts were then stored at –80 °C.

#### 2.4. Preparation of fresh organ samples

One to three grams of each organ sample were pooled and homogenised with sterile sand, and re-suspended in 2–3 ml diethyl pyrocarbonate (DEPC)-treated water. After centrifugation the RNA was

extracted as described above. The extracts were stored at –80 °C.

#### 2.5. PCR amplification

##### 2.5.1. Primers

The general screening for FCoV was carried out by RT- and nested (n) PCR as described by Herrewegh et al. (1995) using the primers p205 and p211 for RT-PCR and p204 and p276 for nPCR, respectively. Samples positive in these assays were submitted to further analysis employing oligonucleotide primers, which had been designed in regions of the S-protein gene allowing a differentiation between FCoV types I and II. To improve the sensitivity of the PCR assays, a second round of amplifications (nPCR with primers b, seminested with primers a) was carried out following RT-PCR. The primers were selected with the help of the Primer Designer Program (Scientific and Educational Software, Version 3.0) and are shown in Table 2.

##### 2.5.2. RT-PCR

RT-PCR was carried out as a single-tube assay with a reaction volume of 25 µl (22.5 µl PCR mixture

Table 2  
Details of the RT-, nested and seminested PCR primers for the detection of FCoV types I and II

Name	Sequence	Orientation	Annealing (°C)	Position	Strain (GenBank accession no.)	Product length (bp)
RT-PCR FCoV type I						
fecv1af	5'-caatgccttaggcctgtacc-3'	Sense	60	1024	KU-2 (D32044)	198
fecv1ar	5'-ggctatggaggcagttgtat-3'	Antisense		1221		
fecv1bf	5'-ttgacctgactggctcaac-3'	Sense	60	4238	KU-2 (D32044)	275
fecv1br	5'-cgtccacagagatgccaata-3'	Antisense		4512		
RT-PCR FCoV type II						
fecv2af	5'-cctacagaggtgtgtgtaaca-3'	Sense	60	250	79-1146 (X06170)	124
fecv2ar	5'-cacgtgcattaccagtcta-3'	Antisense		373		
fecv2bf	5'-aggttgttgatgatcatag-3'	Sense	60	469	79-1146 (AR017842)	232
fecv2br	5'-acggtcaagttcgtcaagta-3'	Antisense		700		
Seminested and nPCR FCoV type I						
fecv1anf	5'-cctgtaccatcgtgtgcttaa-3'	Sense	60	1036	KU-2 (D32044)	186
fecv1ar	5'-ggctatggaggcagttgtat-3'	Antisense		1221		
fecv1bnf	5'-tctgcataccactgttactg-3'	Sense	60	4322	KU-2 (D32044)	183
fecv1bnr	5'-gagatgccaatagacttgac-3'	Antisense		4504		
Seminested and nPCR FCoV type II						
fecv2anf	5'-ggtgtgtgtaactgctcta-3'	Sense	60	258	79-1146 (X06170)	116
fecv2ar	5'-cacgtgcattaccagtcta-3'	Antisense		373		
fecv2bnf	5'-aggaagtgtgtgactcta-3'	Sense	60	496	79-1146 (AR017842)	151
fecv2bnr	5'-gtccatacaagactgtaata-3'	Antisense		646		

and 2.5  $\mu$ l template) using a commercially available kit (Access RT-PCR System, Promega, Madison, WI, USA). The  $MgSO_4$  concentration was optimised at 1 and 2 mM using the primers fecv1 and fecv2, respectively. Negative samples were re-tested by employing the One Step RT-PCR Kit from Qiagen (Valencia, CA, USA). Cyclor schemes were carried out following the instructions of the manufacturers.

### 2.5.3. nPCR and seminested PCR

An amount of 2.5  $\mu$ l of the RT-PCR product was added to 22.5  $\mu$ l of the master mix for nPCR and seminested PCR, respectively, containing 10 mM Tris-HCl (pH 9 at 25 °C), 50 mM KCl, 0.1% Triton X-100, 200  $\mu$ M each 2'-deoxynucleoside 5'-triphosphate, 1.5 mM  $MgCl_2$ , 1.25 U Taq polymerase and 40 pM of each primer. Forty-five cycles of denaturation at 94 °C, primer annealing at 60 °C and primer extension at 72 °C, 30 s each, were employed.

### 2.6. Precautions to minimise the risk of contamination

As the possibility of false positive results due to carryover of amplification products in particular during nPCR cannot be ruled out, a number of precautions were taken to minimise the risk of contamination. These included the physical separation of all PCR procedures, the use of at least four negative controls of RNase free water for each assay and of three or more primer pairs for each sample. The RT-PCR amplification product was added to the master mix for the nPCR in a laboratory specifically installed for this purpose. Finally sequence analysis of 15 amplification products served as an additional control.

### 2.7. Analysis of amplified DNA

Twenty microlitres of each PCR product were analysed by electrophoresis in a 1% agarose gel for 1 h 10 min at 90 V, and visualised by ethidium bromide staining. The 100 bp ladder (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) served as molecular weight marker. Bands were visualised with UV illumination and photographed using the Eagle Eye TM II UV gel imaging system (Stratagene, La Jolla, CA, USA).

Sequence analysis was performed after gel extraction of the amplified product (QIA Quick Gel Extraction Kit, Qiagen, Valencia, CA, USA) and sequencing PCR (ABI PRISM Big Dye<sup>TM</sup> Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer, Alameda, CA, USA) using the sequence analyser ABI PRISM 310 Genetic Analyser (PE Applied Biosystems). Partial nucleotide sequences (a stretch of 108 bp within the S-protein gene region) of selected 11 type I and 2 type II positive samples, as well as one of the five samples which had tested positive for both types, were determined; their alignment is shown in Fig. 1.

### 2.8. Specificity of PCR

Extracts of cell culture supernatants from five different FCoV-strains (type I: FIPV KU2, FIPV NW1; type II: FIPV 79-1146, FECV 79-1683, FIPV DF2) were submitted to RT-PCR, nested and seminested PCR employing the primers fecv1a, fecv1b, fecv2a and fecv2b.

### 2.9. Sensitivity of PCR

Gel extracts of the strains FIPV KU2 and FIPV 79-1146, containing 8.85 and 6.69 pmol/ $\mu$ l DNA, respectively, obtained after RT-PCR with the primers fecv1b and fecv2b, were diluted in RNase free water with a concentration of 1% tRNA, and served as template for nPCR.

### 2.10. Antibody titres

As far as antibody titres had been recorded in the case histories, they were compared to the PCR results.

### 2.11. Pathology

The results of the pathological examination were analysed according to the following criteria: during section the presence and amount of FIP typical effusion (low, medium, high amount) and of FIP suspicious granulomas and pyogranulomas (granulomas yes, no and localisation) were recorded. The subsequent histopathological examination confirmed the diagnosis FIP only in the presence of the typical vasculitis with central necrosis and perivascular

<b>FCoV-1 KU2</b>	4373	<b>G T T T T G G T T G T G T T G G C A G T T G T T G T C A T T C T C T T T G T A G</b>	
FCoV-1 Au 1	1	. C . C . . . . . C . . . . . C . . . . . C . . C T .	
FCoV-1 Au 2	1	. C . . . . . C . . . . . C . . . . . C . . C . .	
FCoV-1 Au 3	1	. C . . . . . C C . . . . A . . C . . . . A . . . . . C . .	
FCoV-1 Au 5	1	. C . . . . . C . . . . A . . . . . C . C . . . . G . . . .	
FCoV-1 Au 4	1	. C . . . . . C . . . . . C . . . . . C A . . . . . C . . C . .	
FCoV-1 Au 6	1	. C . . . . . C . . . . A . . C . . . . A . C . . . . C . .	
FCoV-1 Au 7	1	. C . . . . C . . . C . . . A . . . . . C . . C . . . . C . .	
FCoV-1 Au 8	1	. C . . . . . C . . . . A . . C . . . . A . . . . . C . . C . .	
FCoV-1 Au 9	1	. C . . . . . C . . . . A . . C . . . . A . C . . . . C . . C . .	
FCoV-1 Au 10	1	. C . . . . . C . . . . . C . . . . . C A . . . . . C . . C . .	
FCoV-1 Au 11	1	. C . . . . . C . . . . . C . . . . . C A . . . . . C . . C . .	
FCoV-1 DI	1	. . . . . C . . . . A . . . . . A . . . . . . . . . . . . . . .	
<b>FCoV-2 79-11464337</b>		<b>. C A . A . . . . . T . A . . A . . . . . . . . . . . C . . . . A . A . . . . .</b>	
FCoV-2 Au 1	1	. C A . A . . . . . C T . A . T A . . . . . . . . . . . C . . . . A . . . . . .	
FCoV-2 Au 2	1	. C A . A . . . . . C T . A . . . . . A . . . . . . . . . . . C . . . . A . . . . . .	
FCoV-2 DI	1	. C A . A . . . . . T . A . . . . . A . . . . . . . . . . . C . . . . A . . . . . .	
<b>CCV-INSAVC-1 4770</b>		<b>. . A . C . . G . . . T . A . . A . . C . . . . . . . . . . . C A . A . . . . .</b>	
<b>FCoV-1 KU2</b>	4414	<b>T A G A A G G C A A T T T G A A A C C T A T G A A C C C A T T G A A A A G G T T</b>	
FCoV-1 Au 1	41	. . C . C . T . . . . . A T . . . C . . . . T . . . . . . . . . . .	
FCoV-1 Au 2	41	. . . . . C . T . . . . . A . . . . . . . . . . T . . . . . . . . . . .	
FCoV-1 Au 3	41	. . . . . C . T . . . . . T T . . . . . . . . . . T . . . . . . . . . . .	
FCoV-1 Au 4	41	. . . . . C . T . . . . . A T . . . C . . . . . . . . . . . . . . . . .	
FCoV-1 Au 5	41	. . . . . . A . . . . . A . . . . . . . . . . T . . . . . . . . . . .	
FCoV-1 Au 6	41	. . . . . C . T . . . . . A T . . . . . . . . . . T . . . . . . . . . . .	
FCoV-1 Au 7	41	. . . . . C . T . . . . . A . . . . . . . . . . T . A . . . . . G . . . .	
FCoV-1 Au 8	41	. . . . . C . T . . . . . A . . . . . . . . . . T . . . . . G . . . . .	
FCoV-1 Au 9	41	. . . . . C . T . . . . . C A T . . . . . . . . . . T . . . . . . . . . . .	
FCoV-1 Au 10	41	. . . . . C . T . . . . . A T . . . . . . . . . . T . . . . . . . . . . .	
FCoV-1 Au 11	41	. . . . . C . T . . . . . A T . . . C . . . . . . . . . . . . . . . . .	
FCoV-1 DI	41	. . . . . A . . . . . G T . . C . . . . T T . . . . . . . . . . . . . . .	
<b>FCoV-2 79-11464377</b>		<b>. . . . . A . . . . . . . . . . A T . . . . . A . . . . . A . . . . . G</b>	
FCoV-2 Au 1	41	. . . . . A . . . . . . . . . . A T . . . . . A . . . . . A . . . . . G	
FCoV-2 Au 2	41	. . . . . A . . . . . . . . . . A T . . . . . A . . . . . A . . . . . G	
FCoV-2 DI	41	. . . . . A . . . . . . . . . . A T . . . . . A . . . . . A . . . . . G	
<b>CCV-INSAVC-1 4810</b>		<b>. . . . . G . C . . . . . . . . . . G T . . . . . . . . . . T . . . . . A . . . .</b>	
<b>FCoV-1 KU2</b>	4454	<b>C A C A T T C A T T A A C T A G A C G A T T T A T G G A</b>	<b>IDENTITY</b>
FCoV-1 Au 1	81	. . . . . A .	<b>86 %</b>
FCoV-1 Au 2	81	. . . . . A .	<b>91 %</b>
FCoV-1 Au 3	81	. . . . . A .	<b>88 %</b>
FCoV-1 Au 4	81	. . . . . A .	<b>89 %</b>
FCoV-1 Au 5	81	. . . . . A .	<b>91 %</b>
FCoV-1 Au 6	81	. . . . . A .	<b>88 %</b>
FCoV-1 Au 7	81	. . . . . A .	<b>87 %</b>
FCoV-1 Au 8	81	. . . . . A .	<b>90 %</b>
FCoV-1 Au 9	81	. . . . . A .	<b>86 %</b>
FCoV-1 Au 10	81	. . . . . A .	<b>89 %</b>
FCoV-1 Au 11	81	. . . . . A .	<b>89 %</b>
FCoV-1 DI	81	. . . . . A .	<b>89 %</b>
<b>FCoV-2 79-11464417</b>		<b>. . T G . C . . C . . . . T T . A A G . . A . G . A T</b>	<b>73 %</b>
FCoV-2 Au 1	81	. . T G . C . . . . . T C . A A . . A A . . C T C	<b>73 %</b>
FCoV-2 Au 2	81	. . T G . C . . . . . T C . A A . . A A . . C T C	<b>74 %</b>
FCoV-2 DI	81	. . T G . C . . . . . A . T C . A A . . A A . . C T C	<b>75 %</b>
<b>CCV-INSAVC-1 4850</b>		<b>. . T G . . . . C . G . A . T C . A A . . G . T A A . T</b>	<b>72 %</b>

Fig. 1. Multiple alignment of partial nucleotide sequences (108bp) of the S-protein gene region of Austrian FCoV type I (FCoV-1 Au 1–11, GenBank accession no. AF533338–AF533348) and type II (FCoV-2 Au 1–2, GenBank accession no. AY156723–AY156724) positive samples and of one sample with a double infection (FCoV-1 DI and FCoV-2 DI). Reference sequence: FCoV-1 strain KU2 (GenBank accession no. D32044; nt. pos. 4373–4480); also included in the alignment: a reference strain for FCoV-2 (79-1146; GenBank accession no. X06170; nt. pos. 4337–4444) and for canine coronavirus (INSAVC-1; GenBank accession no. D13096; nt. pos. 4770–4877). Light grey-underlay: FCoV type I strains; white: FCoV type II strains; dark-grey underlay: canine coronavirus.

infiltration with plasma cells, macrophages, lymphocytes and neutrophils.

### 3. Results

#### 3.1. Breed, gender and age of the FIP-diseased cats

General data of 154 FIP-diseased cats were analysed and compared to those of 1600 cats without FIP symptoms examined during the same period of time (1997–2000) at the Institute of Pathology and Forensic Veterinary Medicine of the University of Veterinary Medicine in Vienna (Table 1). The statistical examination showed that the incidence of FIP was significantly higher among males versus females ( $P = 0.035$ ), among purebred versus domestic short hair cats ( $P = 0.000$ ) and among young animals up to 1 year ( $P = 0.000$ ). A significantly greater number of males among FIP-diseased cats was found in the age category 0–1 year ( $P = 0.04$ ), but in cats older than 1 year this trend could not be observed ( $P > 0.05$ ).

#### 3.2. Incidence of types I and II

While 6 of the 65 PET samples tested negative, nucleic acid could be detected in all 29 fresh organ samples with the primers described by Herrewegh et al. (1995). The differentiation of the two types was accomplished in 47 of the 59 PET samples which tested positive for FCoV (the PCR result of one additional sample was questionable) and in 27 out of 29 fresh organ samples (one additional questionable result).

In total, a differentiation was possible in 74 samples. Among these, 64 (86%) tested positive for type I, 5 (7%) for type II and 5 (7%) for both types I and II (Table 3).

Of the 69 samples positive for type I, 68 tested positive with the fecv1b primers, 25 with both the fecv1a

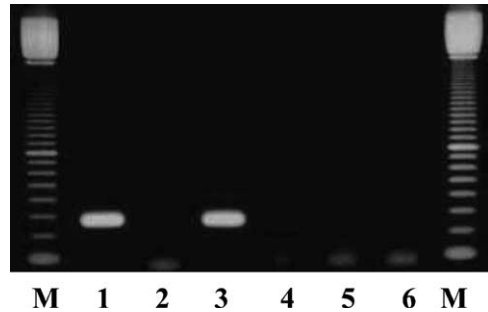


Fig. 2. RT-PCR amplification of cell culture supernatants of five different FCoV strains with the primers fecv1b. Only FCoV type I strains were amplified. M: 100 bp ladder (Amersham Pharmacia), lane 1: strain KU-2, lane 2: negative control, lane 3: strain NW1, lane 4: strain 79-1146, lane 5: strain 79-1683, lane 6: strain DF2.

and fecv1b primer pairs, and 1 with the fecv1a primers only. Of the 2 samples with a questionable PCR result, 1 tested questionably positive with the fecv1a primer pair and negative with the fecv1b primers, the second one vice versa. Of the 10 samples positive for type II, 1 tested positive employing the fecv2a primers and 9 using the fecv2b primers. Of these results 60 (58%) were already achieved after RT-PCR.

#### 3.3. Specificity of the primers

Extracts of cell culture supernatants of five different FCoV strains, the type I strains FIPV KU2 and FIPV NW1, and the type II strains FIPV 79-1146, FECV 79-1683 and FIPV DF2, were subjected to RT-PCR and nested or seminested PCR with the primer pairs fecv1a, fecv1b, fecv2a and fecv2b.

Employing the primers fecv1a and fecv1b, the type I strains KU2 and NW1 showed amplification products of the estimated size, whereas the type II strains 79-1683, 79-1146 and DF2 tested negative (Fig. 2, primers fecv1b). With the primers fecv2a and fecv2b the strains 79-1683, 79-1146 and DF2 tested positive

Table 3  
Incidence of FCoV types I and II in PET and organ samples

	Negative	FCoV positive	Type I positive	Type II positive	Types I and II positive
PET ( $n = 65$ )	6	59	41 (87.2%)	3 (6.4%)	3 (6.4%)
Organ samples ( $n = 29$ )	0	29	23 (85.2%)	2 (7.4%)	2 (7.4%)
Total ( $n = 94$ )	6	88	64 (86.4%)	5 (6.8%)	5 (6.8%)



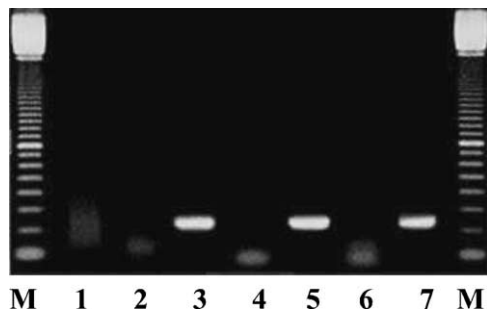


Fig. 3. RT-PCR amplification of cell culture supernatants of five different FCoV strains employing the primers fecv2b. Only FCoV type II strains were amplified. M: 100 bp ladder (Amersham Pharmacia), lane 1: strain KU-2, lane 2: strain NW1, lane 3: strain 79-1146, lane 4: negative control, lane 5: strain 79-1683, lane 6: negative control, lane 7: strain DF2.

whereas the type I strains remained negative (Fig. 3, primers fecv2b).

### 3.4. Sensitivity of the PCR assays

Gel extracts of the strains FIPV KU2 and FIPV 79-1146, obtained after RT-PCR with the primer pairs fecv1b and fecv2b, tested positive in the nested PCR assays up to a dilution of  $10^{-6}$  and  $10^{-9}$ , respectively.

### 3.5. Antibody titres

Antibody titres to FCoV were known from the case history for 25 cats, 20 of them had titres of  $\geq 1:400$ , 3 of 1:100, 1 of 1:10 and 1 was indicated as serologically negative.

In the group of cats with FCoV antibody titres of  $\geq 1:400$  twelve tested positive for FCoV nucleic acid of type I and were negative for type II, and one was positive for type II but negative for type I; for seven cats a differentiation between the two types could not be achieved. All three cats with titres of 1:100 tested positive for type I and negative for type II. The single cat with a titre of 1:10 and the sero-negative cat tested both negative for FCoV nucleic acid.

### 3.6. Sequence analysis

Partial nucleotide sequences of the S-protein gene of 11 type I and 2 type II positive samples as well as of one sample positive for both types were deter-

mined. The sequences were compiled (resulting in a readable stretch of 108 bp) and aligned using the sequence of the type I strain FIPV KU2 as a reference. In the alignment, also the corresponding sequences of the FCoV type II reference strain 79-1146 and of the CCV reference strain INSAVC-1 were included. The analysis of the samples revealed a nucleotide identity of 86–91% for the type I specimens, and of 73–75% for the type II samples, respectively, in reference to the type I strain FIPV KU2 (Fig. 1) and of 77 and 78% for the type II specimens in reference to the CCV strain INSAVC-1.

### 3.7. Pathologic examination

Histopathologic examination exhibited no differences related to the type of FCoV detected. Both types were found in effusive and non-effusive FIP as well as in cases with signs of both forms; a statistical evaluation was not possible due to the small number of type II positive samples.

## 4. Discussion

### 4.1. Breed, gender and age of the cats with FIP

The comparison of the two groups, cats with FIP ( $n = 154$ ) and cats without FIP symptoms ( $n = 1600$ ) examined in the years 1997–2000 at the Institute of Pathology and Forensic Veterinary Medicine of the Veterinary University in Vienna showed significant differences. The statistical evaluation using the  $\chi^2$ -test of the parameters breed, gender and age in the two groups showed that the incidence of FIP was significantly higher among purebred cats, males, and among cats 1 year of age or younger.

The percentage of purebred cats in FIP-diseased cats was more than twice as high as in the comparative group (33.6% versus 13.5%,  $P \leq 0.001$ ). These findings are sustained by earlier studies (Pedersen, 1983). Foley and Pedersen (1996) observed a higher susceptibility for FIP in purebred cats when a first degree relative succumbed to FIP. Due to inbreeding a genetic predisposition may have evolved in certain breeds of cats, which may allow FCoV to propagate more efficiently in these cats than in cats with a wider genetic history.

In the group of the cats with FIP, the majority was male (62.4%), only 37.6% were female. Although in the comparative group the percentage of males was slightly higher as well (53.4% males versus 46.6% females), the difference between the groups was significant ( $P \leq 0.05$ ). These findings are in contrast to Pedersen (1976a), who did not find a sexual predisposition, but in accordance with Potkay et al. (1974) and Binder and Hartmann (2000), who also reported a higher incidence of FIP among males.

The majority of the cats with FIP was 1-year-old or younger (52.1%), in the comparative group only 33.1% were in this age class. Addie and Jarrett (1992a) found as well as Scott (1991) a higher incidence of FIP in cats of up to 1 year.

When comparing the incidence of males and females in the age groups 0–1 year and older than 1 year between the cats with and without FIP, we found that among younger cats the incidence of males was significantly higher in the cats with FIP than in the comparative group. The role of sex-specific differences in the immune system, in particular the cell mediated immunity and the importance of these factors in neutered animals (hormonal influence) are still not clear.

#### 4.2. PCR results

In a total of 93.6% of the samples, FCoV nucleic acid could be detected. Only six PET samples tested negative, whereas all fresh organ samples tested positive.

In respect to the expected lower RNA concentration in the PETs due to the formalin fixation procedure on one hand and due to the long storage time (2–3 years) on the other hand, we chose primers which amplified, compared to those employed by Posch et al. (1999, 2001), a smaller segment of the viral genome. With these primers we achieved a differentiation of the two types in 47 of 65 PET specimens and in 27 of 29 fresh organ samples, in addition two samples exhibited a questionable PCR result. As expected, the percentage of positive PCR results was lower in the PET samples than in the fresh organ samples.

#### 4.3. Specificity and sensitivity of primers

Specificity was tested on five different FCoV strains with four different primer pairs (Figs. 2 and 3, primers

fecvb). We found no false positive results and all amplification products showed bands of the expected size. Despite the use of different primer pairs, in some samples the PCRs remained negative, probably due to the variability in the S-protein gene of FCoV.

The oligonucleotide primers employed in the PCR assays exhibited high sensitivity, the fecv1b primers proved to amplify specific nucleic acid up to a dilution of  $10^{-6}$ , and the fecv2b primers showed amplification even up to a dilution of  $10^{-9}$ . Since the original RNA concentration was similar in both samples, these findings indicate a higher sensitivity for the detection of type II viruses. Thus, in those samples, in which the differentiating PCR was unsuccessful, FCoV type I may predominate as well. On the other hand, due to the lower sensitivity of the type I PCR, we cannot exclude a causative involvement of type I in the type II positive cats.

#### 4.4. Incidence of the types I and II

Of the 74 samples in which a differentiation was achieved, 64 (86%) tested positive for type I, 5 (7%) for type II and 5 (7%) for both types I and II, thus identifying type I as the causative agent in the majority of the FIP cases we examined. Whereas Posch et al. (1999, 2001) identified type II in 45% (including those samples with both types) of FCoV-positive cats without FIP symptoms, we found type II only in 14% of cats with FIP involved, among them 7% showing a double infection with both types I and II.

These findings are in contrast to the results of Hohdatsu et al. (1992) in Japanese cats, in which none of the healthy cats tested positive for type II, whereas among the chronically diseased cats without FIP symptoms over 10% and among the FIP-diseased cats even more than 30% were infected with FCoV type II.

Due to their close antigenic relationship in the S-protein gene and the need to choose primers from this region for a possible differentiation between the two FCoV types, an infection with CCV would also have been detected (data not shown). Therefore, a causative involvement of CCV in the type II positive FIP cases cannot be ruled out in this study. The importance of CCV for the cat still remains unclear (Barlough et al., 1985; McArdle et al., 1992), but the possible recombination between FCoV type I

and CCV (Herrewegh et al., 1998; Horzinek, 2000) requires further investigation, in particular the role of CCV in double infections with both FCoV types observed especially in multi-cat households.

The temperature-sensitive FIPV strain used as FIP vaccine is a type II strain (FIPV DF2). Regarding the fact that the majority of the FIP cases we examined was due to type I, the question arises whether this fact contributes to some of the observed vaccine failures, and whether the inclusion of also a type I strain in the vaccine should be considered.

#### 4.5. Results of sequencing

The sequencing results show even in the very short region, which had been sequenced, a clear discrimination between FCoV type I, FCoV type II and CCV strains (Fig. 1). The 11 partial sequences of the Austrian FCoV type I samples show significant differences, compared to the reference strain KU2, which resulted in identity rates of (only) 86–91% to the reference strain; also within the Austrian FCoV type I samples several nucleotide changes can be noticed, indicative of a quite high mutation rate. The FCoV type II samples form an own group with an identity to the FCoV type I reference strain of only 73–75%, respectively. CCV exhibits an identity to FCoV-1 KU-2 of 72%; its much closer relationship to FCoV type II than to FCoV type I can nicely be observed by the similarity of several nucleotide changes of FCoV type II and CCV. These findings support the observation that FCoV type II may arise from recombinations with CCV (Herrewegh et al., 1998). Nonetheless, CCV also exhibits several unique nucleotide changes. The sequencing data of one of the five samples showing double infections (Fig. 1) demonstrate clearly the plausibility of such co-infections.

#### 4.6. Antibody titres

Among the cats with known antibody titres, all cats positive for FCoV nucleic acid showed antibody titres of 1:100 or higher.

#### 4.7. Pathologic examination

Neither during section nor in the histopathologic examination any differences related to the FCoV type

detected could be identified. In the group of the type I positive cats 58% showed signs of both forms (effusive and non-effusive) of FIP, followed by 28% with non-effusive and 14% with effusive FIP. In the type II positive samples all forms of FIP were represented as well.

These findings do not point towards a pathogenetic importance of the receptor-specificity of type II (Hohdatsu et al., 1998), but emphasises the role of the immune response and the genetic predisposition of the individual in the outbreak of the disease.

## 5. Conclusion

Our findings suggest an involvement of each of both FCoV types in FIP which is in accordance with earlier reports that both types of FCoV are able to cause FIP; they also correlate with the results obtained in healthy FCoV-infected cats, supporting the predominance of FCoV type I infections in both FCoV-infected healthy and FIP-diseased cats. FCoV type II, the probable recombination between type I and canine coronavirus, was involved in 14% of the FIP cases investigated. It has to be assumed that these recombinations occur in the field and therefore contact with dogs excreting canine coronavirus may play a role in the emergence of new type II FCoV. However, the samples positive for FCoV type II need further investigations with respect to their relationship and even differentiation to CCV. Special interest should also be paid to cats with double infections concerning the role of field infections with CCV and their role in the development of FIP. Finally, no differences in the histopathological changes were found related to the FCoV type detected.

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