M gene analysis of atypical strains of feline and canine coronavirus circulating in an Austrian animal shelter

V. BENETKA, J. KOLODZIEJEK, K. WALK, M. RENNHOFER, K. MÖSTL

Coronavirus-positive samples of faeces collected in an Austrian animal shelter from 12 cats and 10 dogs were analysed by reverse transcriptase-PCR with primers amplifying a segment of the M protein gene, and by sequence analysis. In addition, the samples were subjected to S gene typing, using primers that differentiated between feline coronavirus (FCoV) types I and II. A phylogenetic analysis of the M gene sequences revealed not only clearly segregating canine coronavirus (CCoV) in the dogs, typical CCoV sequences and the recently described FCoV-like CCoV, but also at least two genetic clusters of FCoV in the cats, one species-specific, the other more closely related to FCoV-like CCoV. The M gene sequences of these new feline strains had at most 88 per cent identity with the FCoV-like CCoV strain 259/01 and only up to 85 per cent with any FCoV sequence available in GenBank. In the phylogenetic tree they occupy an intermediate position between feline and canine coronaviruses.

THE genus *Coronavirus* is divided into three groups; group 1 includes, among others, the genetically very similar species, canine coronavirus (CCoV), feline coronavirus (FCoV) and transmissible gastroenteritis virus (TGEV) including porcine respiratory coronavirus (PRCV). Coronaviruses are widespread in cats and dogs. Epidemiological investigations have shown that up to 90 per cent of shelter cats and 10 to 70 per cent of cats in single and multicat households were seropositive (Pedersen 1976, Addie and Jarrett 1992a, b, Sparkes and others 1992, Posch and others 2001) and that 20 to 70 per cent of the dog population were seropositive (Möstl and others 1994, Naylor and others 2001b). In multicat households, kennels and animal shelters, the control of coronavirus infections is essential and poses one of the main hygiene problems. Asymptomatic carriers shed the virus for months (or even lifelong) and reinfection is possible after short refractory periods (Addie and Jarrett 2001). Both FCoV and CCoV cause mild to severe diarrhoea, especially in young animals, and in 5 to 10 per cent of infected cats FCoV causes an incurable, fatal immune-mediated disease, feline infectious peritonitis (FIP).

The close antigenic relationship between feline and canine coronaviruses leads to a potential for cross-species infection (Pedersen and others 1978, Horzinek and others 1982, Barlough and others 1984, McArdle and others 1992). In both FCoV and CCoV, interspecies recombinations have been found by sequence analysis in different parts of the viral genome, for example, the S gene, encoding the 'spikes' on the viral surface, and the M gene, encoding the 'membrane' or 'integral membrane' protein. A new FCoV type, designated as type II, has emerged in the field by a recombination (in the region of the S protein gene virus) between the feline type I and CCoV (Herrewegh and others 1998). Epidemiological investigations have shown that FCoV type I is the predominant type in the field (Posch and others 2001, Addie and others 2003, Benetka and others 2004). Infections with more than one FCoV strain are rare but do occur (Posch and others 2001, Addie and others 2003, Benetka and others 2004). Furthermore, it has been shown that FCoV type I strains bind feline aminopeptidase N (fAPN), a cell surface receptor in the lung, spleen, kidney and gut, whereas type II strains do not bind fAPN. The possible clinical and epidemiological impact of these findings is not clear (Tresnan and others 1996, Benetka and others 2004).

There are also at least two genetic clusters of CCoV in the region of the S gene. Sequence analysis of the S gene of strains of CCoV and field isolates revealed that in this part of the genome most of them are closely related to FCoV, although some CCoV isolates were more closely related to TGEV than to FCoV (Wesseling and others 1994, Horsburgh and Brown 1995, Wesley 1999, Naylor and others 2001a, b, 2002). Recent studies of S gene sequences of field isolates from dogs by Pratelli and others (2004) revealed two types of CCoV; CCoV isolates designated as type I are more closely related to FCoV type I than to typical CCoV (designated as CCoV type II) or to FCoV type II.

Pratelli and others (2000, 2001, 2002a, b, 2003a, b) also analysed M gene sequences of strains of CCoV and showed that in this part of the genome, too, there are two genetic clusters of CCoV. The newly discovered second genetic cluster, the so-called FCoV-like CCoV, segregates clearly from the typical strains and is more closely related to FCoV strains than to CCoV strains. These divergent FCoV-like CCoV strains have emerged particularly in dog kennels and animal shelters. It appears that some of them may be more virulent than typical CCoV strains, causing severe haemorrhagic diarrhoea.

This study was designed to obtain more information about the epidemiology of strains of coronavirus in dogs and cats, and to find out whether interspecies transmissions and recombinations occurred in the field. Coronaviruses isolated from coronavirus-positive samples of faeces collected from dogs and cats in an Austrian animal shelter were investigated with primers that differentiate between FCoV types I and II (Benetka and others 2004), and typical CCoV from FCoV-like CCoV (Pratelli and others 2002b). The M gene amplification products, 409 base pairs (bp) in length, obtained from coronavirus-positive faecal samples from 12 cats and 10 dogs were further investigated by sequence and phylogenetic analysis.

MATERIALS AND METHODS

Sample material and clinical history

Samples of faeces were collected from individual dogs and cats kept in an Austrian animal shelter, planned for approximately 150 dogs and 250 cats, but which was usually overcrowded. The cats and dogs were kept separately, but there was indirect contact through cleaning and feeding personnel. The dogs were kept individually but the cats were kept in groups of 10 or more in 'rooms'. Randomly chosen coronavirus-positive faecal samples, identified by reverse transcriptase-PCR (RT-PCR) with the primers p204 and p276 (Herrewegh and others 1995), from 12 cats and 10 dogs were analysed. Some of the samples were taken from animals on the day they arrived at the shelter, but others were taken from animals that had lived at the shelter for more than five years (Table 1). The clinical history of the 12 cats was recorded at least once,

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V. Benetka, DVM, **J. Kolodziejek,** Dr, **K. Walk, M. Rennhofer,** DVM, **K. Möstl,** DVM, Clinical Virology, Department of Diagnostic Imaging, Infectious Diseases and Clinical Pathology, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria

Correspondence to Professor Möstl

TABLE 1: Age, sex, clinical signs and duration of stay of cats and dogs at a shelter in Austria investigated for feline and canine coronaviruses

Animal	Age/sex	Clinical signs	Duration of stay
Dog			
1	0·6 y/M	Diarrhoea	17 days
2	4 y/M	Diarrhoea	2 days
3	17 y/M	Diarrhoea	1 day
4	7 y/M	Diarrhoea	>2 years
5	1 y/M	Diarrhoea	>1 year
6	<1 y/F	Diarrhoea	2 months
7	>1 y/F	Diarrhoea	1 month
8	>1 y/M	Diarrhoea	2 months
9	>6 y/F	Diarrhoea	>5 years
10	>1 y/M	Diarrhoea	2 days
Cat			
1	0·5 y/F	Respiratory	1 month
2	>1 y/M	Respiratory	1 month
3	3 y/F	Diarrhoea	1·5 months
4	0·8 y/F	Healthy	>9 months
5	3 y/F	Healthy	2·5 months
6	>2 y/F	Ascites, FIP suspicious	>2 years
7	0·5 y/F	Healthy	4 months
8	5 y/M	Healthy	Sampled on arrival
9	3 y/M	Respiratory	Sampled on arrival
10	1 y/M	Diarrhoea, FIP suspicious	Sampled on arrival
11	1 y/M	Healthy	>9 months
12	1 y/F	Healthy	>9 months

M Male, F Female, y Year, FIP Feline infectious peritonitis

when the sample was collected, and the 10 dogs were selected because they had diarrhoea (Table 1).

Sample preparation and RNA extraction

Approximately 10 g of the sample was suspended in 2 to 3 ml diethylpyrocarbonate-treated water and after centrifugation at 3400 *g* for 15 minutes, 140 µl of the supernatant was processed. Samples of RNA were extracted with a commercially available kit (QIAamp Viral RNA Kit; Qiagen) and the extracts were stored at –80°C until analysed by PCR.

S gene- and M gene-targeted RT-PCRs

Each sample was analysed by the following three RT-PCRs: a nested PCR targeting a fragment of the S gene specific for FCoV type I with primer pair 1b (Benetka and others 2004); a nested PCR targeting a fragment of the S gene specific for FCoV type II with primer pair 2b (Benetka and others 2004); and a RT-PCR targeting a fragment of the M gene with primers CCoV1 and CCoV2 (Pratelli and others 2002b). The amplification products obtained after RT-PCR with the primers CCoV1 and CCoV2, amplifying 409 bp of the M gene (Pratelli and others 2002b), were submitted to sequence analysis.

DNA extraction, sequencing PCR and sequence analysis

The amplified DNA obtained after RT-PCR with the primers CCoV1 and CCoV2 (Pratelli and others 2002b) was purified by using a commercially available kit (NucleoSpin Extract; Machery-Nagel) following the manufacturer's instructions, and served as template for a sequencing PCR. This was carried out in a final volume of 20 µl with a ready-to-use sequencing PCR mixture (DNA Sequencing Kit; Applied Biosystems) including 4 pmol of each primer CCoV1 and CCoV2.

For each sample the forward and reverse sequences were determined. The sequencing PCR products were analysed with the ABI Prism 310 Genetic analyser.

TABLE 2: Coronavirus reference strains used for the sequence and phylogenetic analyses of viruses isolated from the dogs and cats at the shelter

Coronavirus strain	GenBank accession number	Description
CCoV INSAVC-1	D13096	CCoV reference strain
CCoV BGF-10	AY342160	CCoV reference strain
CCoV 259/01	AF502583	FCoV-like CCoV (M-gene; Pratelli and others 2002b)
FCoV 79-1146	X56496	Type II
FCoV strain Black	AB086903	Type II
FCoV UCD-1	AB086902	Type I
TGEV strain Purdue	AJ271965	Porcine
PRCV strain 86/137004	X60056	Porcine

CCoV Canine coronavirus, FCoV Feline coronavirus, TFEV Transmissible gastroenteritis virus, PRCV Porcine respiratory coronavirus

Alignments and phylogenetic analysis

All the sequences determined and other feline, canine and porcine M gene sequences obtained from GenBank are shown in Table 2. The alignments were generated by using the program AlignPlus version 4.0 (Scientific and Educational Software), and a phylogenetic analysis was performed by using the PHYLIP programs (Felsenstein 1992) Seqboot (100 replicates) and Dnadist (Kimura 2-parameter method). The phylogenetic relationships were deduced by using the neighbour-joining method. The CCoV strain BGF-10 (GenBank accession number AY342160) served as an outgroup.

RESULTS

Samples from cats

The sequence and phylogenetic analyses revealed two genetic clusters in the 12 samples from cats. One cluster consisted of the samples from cats 5 to 12, which had high sequence identities to typical FCoV reference strains (including both type I and type II) available in GenBank. The second cluster consisted of the samples from cats 1 to 4, which had higher nucleotide identities with FCoV-like CCoV strain 259/01 than with any FCoV or CCoV sequence available in GenBank.

Nucleotide alignments of the M gene sequences There were at least two different genetic clusters in the 12 samples from cats. The M gene sequences of cats 5 to 12 had identities of 92 to 95 per cent with the corresponding sequences of the feline reference strains and only 78 to 86 per cent with the typical CCoV reference strains and the FCoV-like CCoV 259/01; these coronaviruses are designated as typical FCoV. The second cluster consisted of the M gene sequences of cats 1 to 4, and was more closely related to the FCoV-like CCoV strain 259/01 (86 to 88 per cent identity) than to any FCoV strain (81 to 85 per cent). The nucleotide identities of this second cluster did not exceed 88 per cent with any group 1 coronavirus M gene sequence available in GenBank (Table 3).

Amino acid alignments of the M gene sequences The sequences of cats 1 to 4 had sites in their amino acid alignments that were different from all other feline and canine isolates and strains, and they are designated as atypical FCoV. The atypical FCoV M gene sequences of cats 1 to 4 were only 87 to 89 per cent homologous with the feline reference strains, but 90 to 92 per cent homologous with CCoV BGF-10 and 92 to 94 per cent homologous with FCoV-like CCoV 259/01 (Fig 1).

The typical FCoV M gene sequences of cats 5 to 12 were 96 to 99 per cent identical to the corresponding segments of the feline reference strains, but only 86 to 87 per cent identical to typical CCoV BGF-10 and 90 to 91 per cent identical to FCoV-like CCoV 259/01 (Fig 1).

Phylogenetic analysis of the M gene sequences Phylogenetic analysis also revealed the presence of two genetic clusters, consisting of the typical FCoV sequences of cats 5 to 12 and the atypical sequences of cats 1 to 4. Furthermore, in the first group there were two variants, the first consisting of cats 6, 7, 10 and 11 and the strains FCoV Black and 79-1146, and

the second consisting of cats 5, 8, 9 and 12 and strain FCoV UCD-1 (Fig 2).

Samples from dogs

In the samples from the 10 dogs there were also two genetic clusters. One cluster consisted of the samples from dogs 1 to 3, which had high identities to the typical CCoV reference strains. The second cluster consisted of samples from dogs 4 to 10, which had high identities to the recently described FCoV-like CCoV strains such as 259/01.

Nucleotide alignments of the M gene sequences The nucleotide alignments of the 10 samples from dogs revealed at least two different variants of CCoV. The M gene sequences of dogs 1 to 3 were 93 to 96 per cent identical to the equivalent stretch of the typical CCoV reference strains and 94 to 95 per cent identical to the porcine strain TGEV Purdue, but only 87 to 89 per cent identical to the atypical FCoV-like CCoV strain 259/01. The nucleotide sequences of dogs 4 to 10 were 96 to 98 per cent identical to the M gene sequences of FCoV-like CCoV strain 259/01, but only 86 to 91 per cent identical to the typical CCoV reference strains and 86 to 90 per cent identical to TGEV (Table 3).

Amino acid alignments of the M gene sequences When comparing amino acid sequences, the two genetic clusters of CCoV were again evident. In this region the typical CCoV samples of dogs 1 to 3 were only different from strain BGF-10 by one amino acid out of 102 (identity 99 per cent), but the atypical samples of dogs 4 to 10 were different at five locations (identity 95 per cent), all of which were identical to the typical FCoV samples from cats 6 to 12 and the FCoV reference strains. The typical CCoV samples were only 84 to 86 per cent identical to the FCoV reference strains, compared with 89 to 90 per cent for the FCoV-like CCoV samples (Fig 1).

Phylogenetic analysis of M gene sequences Phylogenetic analysis also revealed that there were basically two distinct genetic clusters. The first cluster consisted of sequences from dogs 1 to 3, the CCoV reference strains, PRCV and TGEV, and the second consisted of sequences from dogs 4 to 10 and the FCoV-like CCoV strain 259/01. Furthermore, this second cluster consisted of two variants, one formed by the sequences from dogs 5, 7, 8 and 9 and FCoV-like CCoV strain 259/01, the other by the sequences from dogs 4, 6 and 10 (Fig 2).

TABLE 3: Percentage nucleotide sequence homologies within a 306 base pair section of the M gene of coronaviruses isolated from 12 cats and 10 dogs in a rescue centre and three canine, three feline and one porcine reference strains

	Reference strain						
Animal	CCoV 259/01	CCoV BGF-10	CCoV INSAVC-1	FCoV Black	FCoV UCD-1	FCoV 79-1146	TGEV Purdue
Dog							
1	89	95	95	81	80	85	95
2	89	94	96	81	81	81	94
3	87	93	95	86	85	84	95
4	96	90	88	85	84	85	89
5	98	89	86	83	82	83	87
6	96	90	88	86	84	86	90
7	98	90	87	84	83	84	88
8	98	90	88	83	84	84	88
9	98	89	86	82	82	82	86
10	96	91	89	85	84	85	89
Cat							
1	88	86	83	85	83	85	85
2	87	85	83	84	85	84	84
3	88	86	83	84	83	85	84
4	86	84	81	82	81	84	82
5	83	82	81	92	94	93	80
6	85	82	81	95	94	93	81
7	84	82	80	95	92	93	80
8	84	81	85	94	93	93	86
9	85	82	80	94	94	93	80
10	82	79	78	94	92	92	82
11	86	83	82	95	94	94	83
12	84	82	81	93	94	93	81

CCoV Canine coronavirus, FCoV Feline coronavirus, TGEV Transmissible gastroenteritis virus

Results of S gene typing

S gene typing showed that eight of the 12 cats had a FCoV type I infection, and one sample gave a questionable result for type I; the infection in the other three cats could not be differentiated (Table 4).

In five of the dogs the RT-PCR detecting FCoV type II/CCoV sequences was positive, and the results for two were questionable; the infection in the other three dogs could not be differentiated (Table 4).

Comparison of the results of M gene and S gene typing of the samples from the cats and dogs

When comparing the results of the S gene-based differentiation of FCoV types I and II and the results of the M gene analysis (Table 4), all the typical CCoV samples could

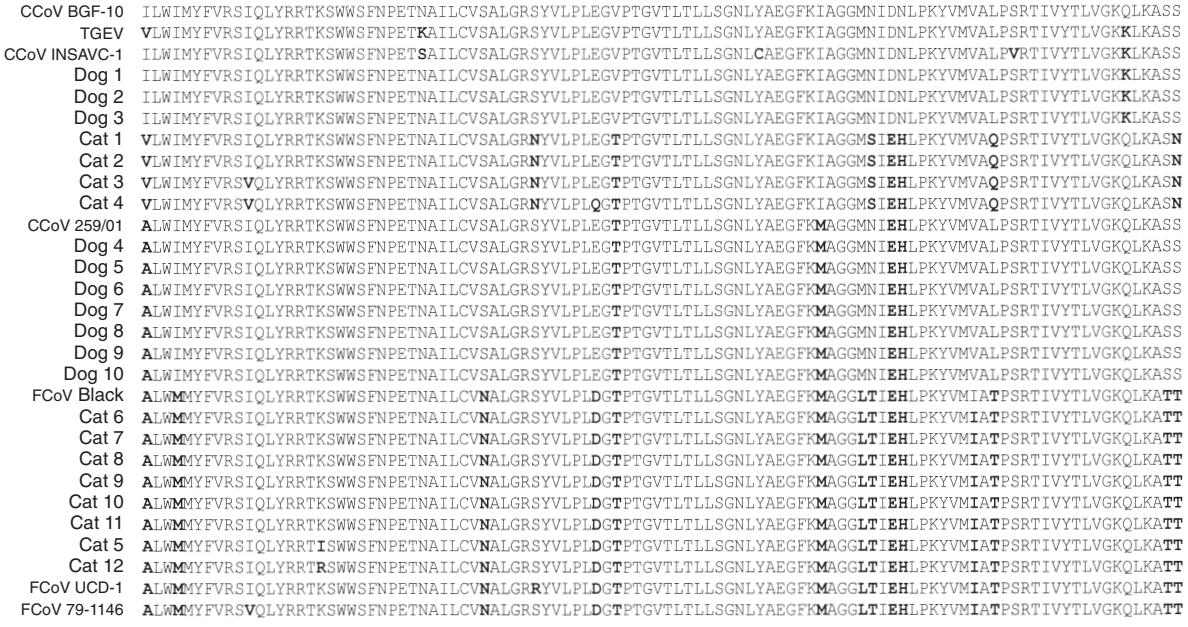

FIG 1: Alignment of 102 amino acids within the M gene of coronaviruses isolated from 12 cats and 10 dogs in a shelter and three canine, three feline and one porcine reference strains; canine coronavirus (CCoV) strain BGF-10 served as a reference strain. FCoV Feline coronavirus, TGEV Transmissible gastroenteritis virus

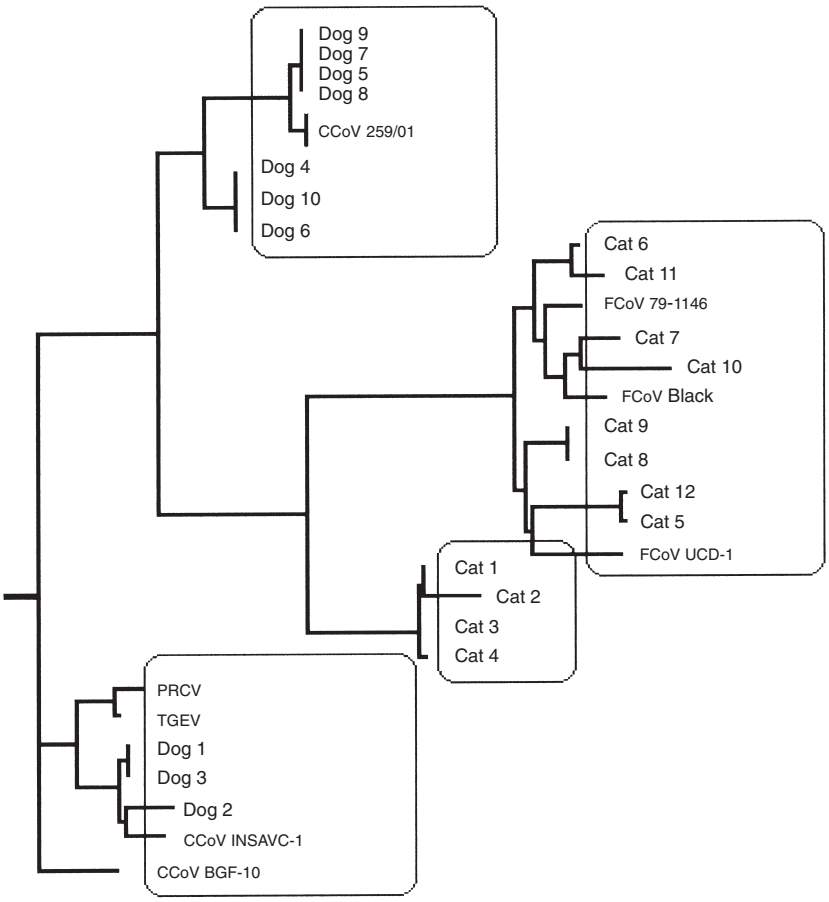

FIG 2: Phylogenetic analysis based on 306 base pairs within the M gene of coronaviruses from 12 cats and 10 dogs in a shelter and three feline, three canine and two porcine reference strains; reference canine coronavirus CCoV BGF-10 strain served as the outgroup. FCoV Feline coronavirus, PRCV Porcine respiratory coronavirus, TGEV Transmissible gastroenteritis virus

be clearly identified as the expected type II. Of the atypical CCoV samples, only two tested clearly positive for FCoV type II, two gave a questionable result for type II and three samples tested negative. All the CCoV samples tested clearly negative for FCoV type I. Of the eight typical FCoV samples, FCoV type I could be identified in seven and the other sample tested negative. In the atypical FCoV samples, a clear differentiation was possible in only one cat, which was positive for FCoV type I, and one sample gave a questionable result for FCoV type I. All the FCoV samples tested clearly negative for FCoV type II.

Clinical signs and duration of stay in relation to the typing results

All 10 dogs had diarrhoea. The three typical CCoV samples were collected from dogs 1, 2 and 3, which had been in the shelter for only one, two and 17 days, respectively. The atypical CCoV samples were from dogs that had been in the shelter for at least a month, except for that from dog 10, which arrived only two days before the sample was taken (Table 1).

The cats showed various clinical signs, for example, respiratory signs, diarrhoea and ascites; only six of the 12 cats were clinically healthy. The atypical FCoV samples originated from cats that had been in the shelter for from one month to over nine months; the typical FCoV samples came from three cats sampled on the day of their arrival and from five cats that had been in the shelter for from 10 weeks to over nine months (Table 1).

TABLE 4: Results of the S gene-based differentiating PCR analyses of the coronaviruses isolated from 10 dogs and 12 cats in a shelter

Animal	Type I	Type II
Dog		
1	–	+
2	–	+
3	–	+
4*	–	–
5*	–	–
6*	–	–
7*	–	?
8*	–	+
9*	–	?
10*	–	+
Cat		
1†	–	–
2†	–	–
3†	?	–
4†	+	–
5	–	–
6	+	–
7	+	–
8	+	–
9	+	–
10	+	–
11	+	–
12	+	–

* Atypical canine coronavirus
† Atypical feline coronavirus,
– Negative, + Positive, ? Questionable

DISCUSSION

M gene analysis showed that eight of the 12 randomly chosen coronavirus-positive faecal samples from the cats in the shelter were positive for typical FCoV, but four, from cats 1 to 4, were infected with an unknown strain of coronavirus; the M gene sequences of these samples, although very similar to both feline and canine coronaviruses, had clearly distinct and unique sites. When comparing sequence identities (Table 3), the divergent samples were most closely related to FCoV-like CCoV and to FCoV, but the highest identity with any group 1 coronavirus did not exceed 88 per cent. In the phylogenetic tree they occupy an intermediate but clearly distinct position between feline and canine strains.

The phylogenetic analysis of the M gene revealed at least two genetic clusters of CCoV in the dogs. The first cluster, of typical CCoV, consisted of the samples from dogs 1 to 3, and the second the clearly segregating samples from dogs 4 to 10, with a further division into two variants: variant 1 is represented by the samples from dogs 5, 7, 8 and 9, which were more closely related to the FCoV-like CCoV strain 259/01 than variant 2, represented by the samples from dogs 4, 6 and 10, which were more closely related to the FCoV strains Black, UCD-1 and 79-1146.

Alignments of the nucleotide and amino acid sequences revealed that, in contrast with typical CCoV, the atypical CCoV samples shared unique sites with strains of FCoV. These results show that there are FCoV-like CCoV strains in Austria, like those described by Pratelli and others (2002a, b, 2003a, b) in Italy, and they underline the possibility that FCoV may be transmitted to dogs and that there may be frequent recombinations between the two virus types. However, as suggested by Pratelli and others (2003b), the possibility of an ancient common ancestor but independent divergence and evolution of these virus types cannot be excluded, but these results and those of previous studies make this possibility seem unlikely.

S gene typing of the samples from the cats showed that at least one of the atypical samples was positive for FCoV type I and therefore conserved in the S gene, but in the other three samples this part of the genome could not be differentiated.

As expected, all the typical CCoV samples tested clearly positive for FCoV type II or more probably CCoV, because in this part of the genome FCoV type II and CCoV are almost identical and therefore cannot be differentiated. But, of seven atypical CCoV samples, only two tested positive for type II, and two gave a questionable result. No infection or double infection of dogs with FCoV type I strains could be detected, and all the samples from dogs tested clearly negative for FCoV type I.

One reason why it was difficult or impossible to do S gene-based typing of both the atypical CCoV and atypical FCoV samples may be that there are as yet unknown variations and recombinations in the S gene, as well as in other sites of the viral genome, which cannot be detected by primers based on typical GenBank sequences. Furthermore, the M gene-based typing did not seem to correlate with the S gene-based typing, a fact that suggests that there may be multifocal recombinations between FCoV and CCoV that are not necessarily correlated, as previously suggested by Herrewegh and others (1998). The sequence analysis needs to be extended to other parts of the genomes of field isolates of CCoV and FCoV, to obtain more detailed information about the predilection sites of such recombinations and mutations.

The results observed with the strains of coronavirus isolated from both the dogs and cats support the suggestion that these viruses may be transmitted between the species, which may lead to a variety of new virus recombinations in both species.

Although only a few animals were investigated, it is remarkable that all the typical CCoV samples originated from animals that had been in the shelter for only a few days and for a maximum of 17 days. On the other hand, with one exception, all the atypical CCoV originated from dogs that had been in the shelter for at least a month. This difference may indicate the presence of shelter-specific virus variants. The atypical FCoV samples originated from cats that had been in the shelter for at least two weeks, whereas the viruses isolated from the samples collected on the day of the cats' arrival were typical FCoV. From a clinical point of view, it was not possible to correlate the clinical signs observed and the virus typing, partly because of the small number of samples collected, and partly because dogs with diarrhoea were selected for investigation.

The clinical aspects of these new variants need to be investigated. The question arises whether these newly discovered atypical FCoV strains are of any clinical importance, in the way that atypical CCoV strains are believed to be.

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V. Benetka, J. Kolodziejek, K. Walk, M. Rennhofer and K. Möstl

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Notes