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Infection of cats with atypical feline coronaviruses harbouring a truncated form of the canine type I non-structural ORF3 gene



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ABSTRACT

Feline and canine coronaviruses (FCoV and CCoV, respectively) are common pathogens of cats and dogs sometimes leading to lethal infections named feline infectious peritonitis (FIP) and canine pantropic coronavirus infection. FCoV and CCoV are each subdivided into two serotypes, FCoV-I/II and CCoV-I/II. A phylogenetic relationship is evident between, on one hand, CCoV-I/FCoV-I, and on the other hand, CCoV-II/FCoV-II, suggesting that interspecies transmission can occur. The aim of the present study was to evaluate the prevalence of coronavirus (CoV)-infected cats according to their contact with dogs and to genetically analyse the CoV strains infecting cats. From 2003 to 2009, we collected 88 faecal samples from healthy cats and 11 ascitic fluids from FIP cats. We investigated the possible contact with dog in the household and collected dogs samples if appropriate. Out of 99 cat samples, 26 were coronavirus positive, with six cats living with at least one dog, thus showing that contact with dogs does not appear as a pre-disposing factor for cats CoV infections.

Molecular and phylogenetic analyses of FCoV strains were conducted using partial N and S sequences. Six divergent strains were identified with the N gene clustering with CCoV-I whereas the 3' end of S was related to FCoV-I. Further analysis on those six samples was attempted by researching the presence of the ORF3 gene, the latter being peculiar to CCoV-I to date. We succeeded to amplify the ORF3 gene in five samples out of six. Thus, our data strongly suggest the circulation of atypical FCoV strains harbouring the CCoV-I ORF3 gene among cats. Moreover, the ORF3 genes recovered from the feline strains exhibited shared deletions, never described before, suggesting that these deletions could be critical in the adaptation of these strains to the feline host.

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1. Introduction

Coronaviruses (CoVs) are enveloped viruses that possess the largest (27–32 kb) single-stranded, positive-sense RNA molecule of all such viruses. According to the novel classification approved by the International Committee of Taxonomy of Viruses, coronaviruses have been divided into four new genera *Alpha*, *Beta*, *Gamma* and *Deltacoronavirus* and each genus is subdivided into different species. Due to their close sequence identity, porcine transmissible gastroenteritis virus (TGEV), canine coronavirus (CCoV) and feline

coronavirus (FCoV) have been regrouped together, henceforth forming the *Alphacoronavirus* 1 species, within the *Alphacoronavirus* genus (Carstens, 2010).

The *Alphacoronavirus* 1 species genome includes 7 open reading frames (ORFs) flanked by 5' and 3' untranslated regions (UTRs). The 5' two thirds of the genome encode proteins involved in RNA replication and transcription (Bredenbeek et al., 1990). Genes encoding the structural proteins are located downstream; these include the spike (S) glycoprotein responsible for virus entry, the small envelope (E) protein, the transmembrane (M) protein and the nucleocapsid (N) protein, which is associated with the viral RNA. The rest of the ORFs encode unknown or less-characterised non-structural proteins. The genome of FCoVs and CCoVs includes two gene clusters encoding non-structural proteins, the ORFs 3a, 3b, 3c (located between the S and E genes) and the ORFs 7a, 7b (downstream of the N gene). These genes are not essential for virus propagation in cell culture but are important in virulence or viral tropism (Chang et al., 2010; Vaughn et al., 1995).

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FCoV infections are common in domestic cats and usually remain subclinical. In some cases, FCoV causes a fatal disease called feline infectious peritonitis (FIP). Two FCoV serotypes exist and can be distinguished by an *in vitro* neutralisation assay (Hohdatsu et al., 1991). In the field, type I FCoVs are predominant (Addie et al., 2003). Type II FCoVs originate from a double recombination between type I FCoV and CCoV, resulting in an FCoV genome with the S gene and the 5' end of the M gene originating from CCoV (Herrewegh et al., 1998).

CCoV are also divided into 2 genotypes, CCoV type I (CCoV-I) and CCoV type II (CCoV-II). CCoV-II was recently subdivided into two subtypes, CCoV-IIa comprising CCoV reference strains and CCoV-IIb, which results from the recombination between CCoV-IIa and TGEV (Decaro et al., 2009). CCoV-I strains were identified in the beginning of the 2000s and were initially designated as FCoV-like strains due to their high sequence homology within the S gene of FCoV-I (Pratelli et al., 2003a,b). Lately, an additional ORF, named ORF3, that has not been detected in CCoV type II and other *alphacoronaviruses*, has been identified in CCoV-I. ORF3 is placed downstream of the S gene and encodes a non-structural glycoprotein of unknown function (Lorusso et al., 2008).

Considering the close genetic relationship between CCoV-I/FCoV-I on one hand, and CCoV-II/FCoV-II on the other hand, interspecific circulation of CoVs between both species is plausible. In 2006, a study performed in an Austrian shelter where cats and dogs lived together, did indeed suggest that some cats were infected with atypical strains related to CCoV-I (Benetka et al., 2006). The present study was designed to provide a greater knowledge of FCoV strains infecting cats from private owners, living or not in close contact with dogs.

2. Materials and methods

2.1. Clinical specimens

Rectal swabs from 88 healthy cats and 11 ascitic fluid samples from cats having clinical signs of a wet form of FIP were collected in France and Romania. The owners were questioned about the eventual presence of dogs in the household. 3 faecal samples from dogs living with cats were thus recovered. Samples were kept frozen at -80°C until tested.

2.2. RNA extraction and RT-PCR assays

RNA from ascitic fluid and frozen faeces was extracted by using the QIAamp viral RNA Mini Kit (QIAGEN). The screening of coronavirus positive samples was performed by using the previously described primers and protocol targeting the highly conserved 3' UTR of the coronavirus genome (Herrewegh et al., 1995). Primers amplifying nucleocapsid N gene (forward, 5'-AACAAACACACCTGGAAGA-3' reverse, 5'-GTGTCATCAAACACATCTGT-3') and ORF3 (forward, 5'-CACTAAACTCAAATGTTGATTC-3' reverse, 5'-TTAAGGATTAACATATTCTA-3') were designated on the basis of sequences available in GenBank. RT-PCR assays were carried out using the *OneStep RT-PCR Kit* (Qiagen). Briefly, the RNA was reverse transcribed at 50°C for 30 min and then amplified by 40 cycles of 1 min of denaturation at 94°C , 1 min of primer annealing at 50°C and 1 min of primer extension at 72°C . The N PCR products were 415 bp in length located between nucleotides position 27466 and 27881 in reference to the sequence of strain FCoV 79-1146. The ORF3 PCR products size were expected around 628 bp, located between nucleotides 16 and 638 in reference to the Elmo/02 CCoV-I strain.

Amplification of the 3' end of the S gene was attempted by using specific FCoV-I and CCoV-I primers and protocols previously described (Addie et al., 2003; Pratelli et al., 2003b; Chang et al., 2012).

2.3. Bank accession numbers

The PCR products were subjected to sequence analysis (Eurofins Company). The partial sequences of the N gene of strains cats-1, -9, -21, -22, -24, -29, -30, -33, -41, -57, -61, -74, -81, -118, -122, -125, -127, -130, -139, -149, -163, -179, -180, -181, -196, -198 and dog-1 have been registered in GenBank under accession numbers JN687591-JN687617. The ORF3 sequences of strains cats-9, -22, -29, -30, -163 and dog-1 have been registered in GenBank under accession numbers JN714195-JN714200. The partial sequences of the S gene of cats-22, -29, -30, -163 and dog-1 have been registered in the European Nucleotide Archive under accession numbers HG325837-HG325840.

2.4. Sequence analysis

The ClustalW2 program (www.ebi.ac.uk/Tools/clustalw2) was used for sequence alignment. Phylogenetic tree was constructed by the Neighbour-Joining method using MEGA version 4, supplying a statistical support with bootstrapping over 1000 replicates (Tamura et al., 2007). Potential signal peptide and N-glycosylation site were determined respectively with Sigcleave (emboss.bioinformatics.nl/cgi-bin/emboss/sigcleave) and NetNGlyc (www.cbs.dtu.dk/services/NetNGlyc/) programs, respectively.

3. Results

3.1. Prevalence of CoV infection in cats living or not with dogs

Over the period 2003–2009, 88 rectal swabs from healthy cats and 11 samples of ascitic fluid from cats that developed a wet form of FIP were collected in France and Romania. The owners were questioned about the presence of dogs in the households. 19/99 (19.2%) of the cats enrolled in the study had been in contact with at least one dog (Table 1).

The presence of coronavirus was assessed by a previously described RT-PCR assay that amplifies the highly conserved 3' end of the viral genome (Herrewegh et al., 1995). All diseased cats were tested positive (Cats-1, -21, -24, -41, -74, -118, -163, -180, -181, -196 and -198) and out of the 88 healthy cats, 15 animals were shedding coronaviruses in their faeces (see Supplementary Table 1). Among those infected animals, 6/26 (23%) were living with at least one dog, which is not statistically different ($p < 0.05$) from the proportion of non CoV-infected cats in contact also with dogs (13/73) (Table 1).

Within our range of action, we sampled rectal swabs from 3 of the dogs living with CoV infected cats and only one (Dog-1), which was in close contact with Cat-22, was shedding coronavirus at the time of sampling.

3.2. Sequence analysis of N gene

Sequence analysis of a fragment of the N gene was performed on the assumption that this ordinarily conserved gene would allow

Table 1
Distribution of coronavirus-infected cats and non coronavirus-infected cats according to the presence or absence of dogs in the household.

	Contact with dogs		Total
	Yes	No	
CoV-infected cats	6	20	26
Non CoV-infected cats	13	60	73
Total	19	80	99

significant discrimination of the phylogenetic relationships between strains. The sequences obtained were compared by phylogenetic analyses with representative FCoV-I/II, CCoV-I/II and TGEV strains retrieved from GenBank. The N sequences clustered into two main clades (Fig. 1). The first clade included FCoV-I/II whereas the second clade is divided into two separate clusters comprising CCoV-I and CCoV-II/TGEV. 20/26 of the feline samples tested fell into the typical FCoV genotype. Half of them were recovered from asymptomatic infections, the others from sick cats but no genetic distinction was evident between N sequences from healthy or diseased cats. With exception given to the sequence from Cat-57, the nucleotide identity of the sequenced N genes to the reference FCoV strains was comprised between 90% and 94% (Fig. 1). Interestingly, 5 sequences recovered from healthy cats (Cats-9, -22, -29, -30 and -179) and one from a FIP cat (Cat-163) segregated within the CCoV-I cluster. The N gene of these strains displayed high sequence identity to CCoV-I reference strains ranging from 91 to 96% but less than 79% with any FCoVs. The N sequence from Dog-1 was also grouped within the CCoV-I cluster (Fig. 1).

3.3. Sequence analysis of the 3' end of the S gene

As the S gene sequences have been widely used to genotype FCoVs and CCoVs and more recently to discriminate virulent FCoV strains leading to FIP, we investigated the 3' end sequence of S with primers specific to CCoV-I and to FCoV-I strains (Addie et al., 2003; Pratelli et al., 2003b; Chang et al., 2012). By using CCoV-I primers, only the S fragment from Dog-1 was amplified and sequenced, confirming its highest sequence homology with CCoV-I over 95% (Fig. 2).

Amplification of the 3' end of S was successful with specific FCoV-I primers for 14/20 cats samples with FCoV-I N genes and for 4/6 samples with N gene clustering with CCoV-I (Fig. 2.). Assays with specific FCoV-II primers failed. Sequence analyses confirmed that the amplified S fragments from Cats -22, -29, -30 and -163, whose N genes clustered with CCoV-I strains, shared the highest sequence identity with prototype or field FCoV-I strains, ranging from 89 to 97.9% and less than 86.2% with prototype CCoV-I strains (Fig. 2). In addition, the sequence from Cat-163, which died from FIP, exhibited a Leucine residue at position 1058, recently described as virulence

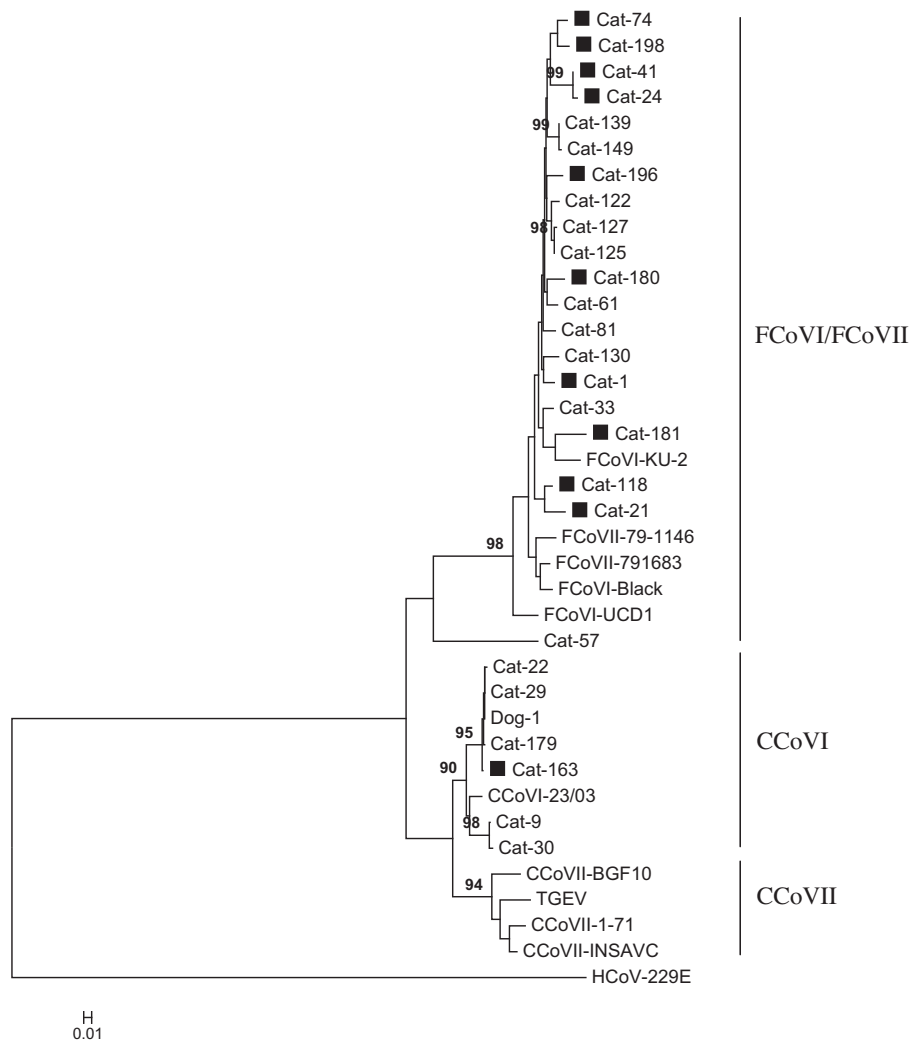


Fig. 1. Phylogenetic tree based on partial nucleotide sequences of the N gene. A neighbour-joining tree was constructed using the MEGA 4.1 software (Tamura et al., 2007). The N gene of human coronavirus 229E (HCoV-229E) serves as an outgroup. A bootstrap analysis of 1000 replicates was performed and values above 90% are indicated on the branches. Horizontal branches are drawn to scale; the scale bar represents 0.1 nucleotide substitutions per site. The following reference strains were used for phylogenetic tree construction (GenBank accession numbers are reported in parentheses): FCoV-79-1683 (AB086904), FCoV-79-1146 (DQ010921), FCoV-UCD1 (AB086902), FCoV-Black (EU186072), FCoV-KU-2 (AB086881), CCoV-1-71 (JQ404409), CCoV-BGF10 (AY342160), CCoV-INSAVC (D13096), CCoV-23/03 (AY548235), TGEV (AJ271965), HCoV-229E (DQ2439391). Sequences marked with ■ were amplified from FIP animals.

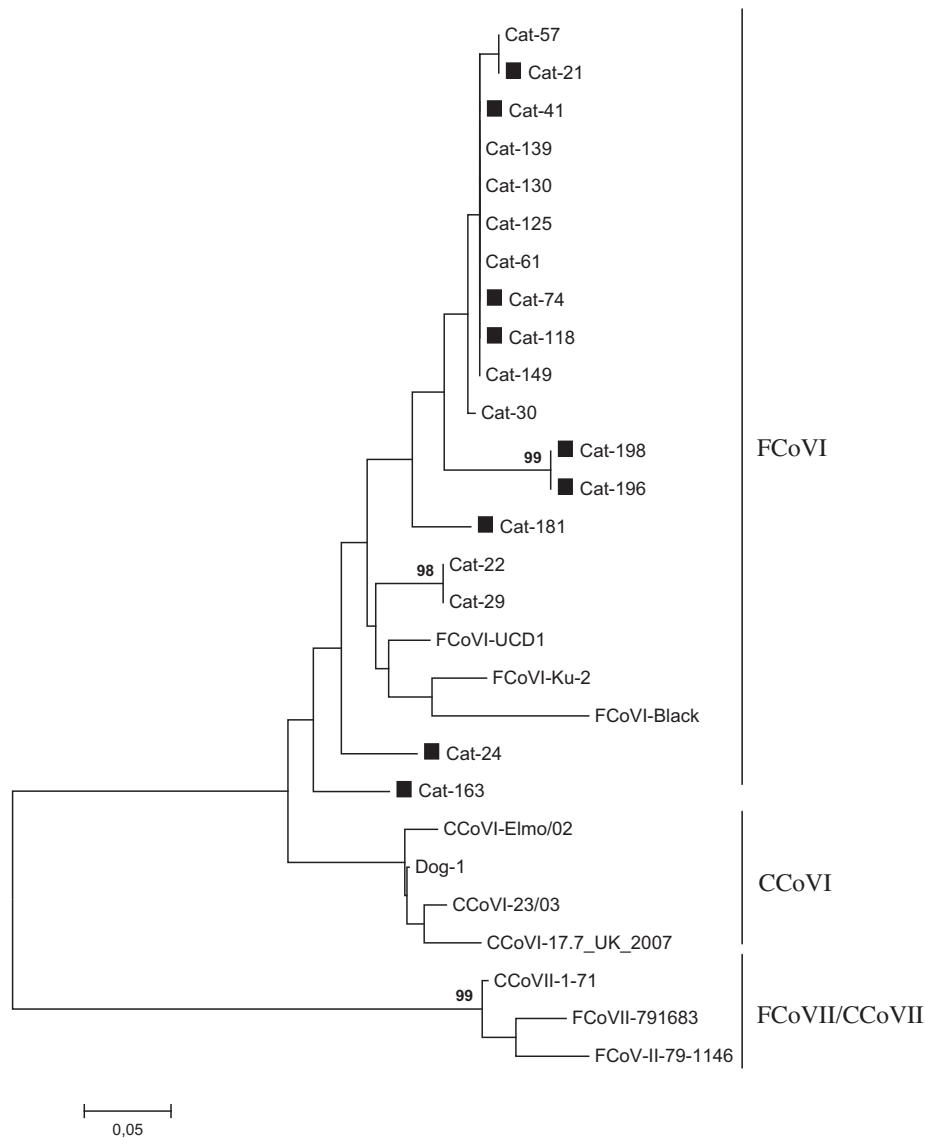


Fig. 2. Phylogenetic tree based on partial nucleotide sequences of the 3' end of the S gene. A neighbour-joining tree was constructed using the MEGA 4.1 software (Tamura et al., 2007). A bootstrap analysis of 1000 replicates was performed and values above 90% are indicated on the branches. Horizontal branches are drawn to scale; the scale bar represents 0.05 nucleotide substitutions per site. The following reference strains were used for phylogenetic tree construction (GenBank accession numbers are reported in parentheses): FCoV-Black (EU186072), FCoV-UCD1 (AB088222), FCoV-KU-2 (D32044), CCoV-23/03 (AY307021), CCoV-Elmo/02 (AY307020), CCoV-17.7_UK_2007 (JX035860), FCoV-II-79-1146 (DQ010921), FCoV-II-791683 (AB086904), CCoV-II-1-71 (JQ404409). Sequences marked with ■ were amplified from FIP animals.

marker of FCoV-I strains (Chang et al., 2012). The other S sequences retrieved from FIP infections also harbour the same mutation.

3.4. Amplification of the ORF3 gene and sequence analysis

Recently, a novel accessory gene named ORF3, located between the end of the S gene and the ORF3a gene, was discovered in CCoV-I strains. This gene is absent in all other *alphacoronaviruses* studied so far. To further characterise feline coronaviruses harbouring an N gene related to CCoV-I, we attempted to amplify the ORF3 gene by RT-PCR. Thus, ORF3 was detected in the Dog-1 sample, and in all but one cat (Cat-179) harbouring an N gene related to CCoV-I. Conversely, the attempt to detect ORF3 in a panel of strains with a feline N gene was unsuccessful.

The sequence comparison of the ORF3 amplicons revealed 85–95% identity with ORF3 nucleotide sequences from the CCoV-I reference strains. The sizes of the amplicons were shorter than the reference sequences. ORF3 sequences from Dog-1 and Cat-22 were

595 bp in length, sequences from Cats-9, -29 and -30 were 568 bp in length and the sequence from Cat-163 was only 541 bp in length, instead of 623 bp for the reference ORF3 sequences deposited in GenBank. By nucleotide alignment with sequences available from GenBank, two deletions, never described before, were identified within the ORF3 gene isolated from cats and Dog-1 (Fig. 3A). The first deletion, located between nucleotides 262 and 289, preserved the reading frame of ORF3 and resulted in the loss of 9 amino acid (aa) starting at the aa 88 (Fig. 3B). This deletion was only observed in Cats-9, -29, -30 and -163. The second deletion was common to all analysed ORF3 sequences, even in the ORF3 gene from Dog-1, which lived with Cat-22. It comprised nucleotides 461–482 and introduced an early stop codon at the aa 159 (Fig. 3B). The ORF3 retrieved from Cat-163 is shortened at the 3' end of the gene.

Computer analysis predicted that the deleted ORF3 would encode a truncated gp3 protein of 149 or 158 aa, while the gp3 protein comprises 207 aa in CCoV-I isolates studied so far (Fig. 3B). All of the truncated gp3 proteins retained the 14-aa signal peptide and

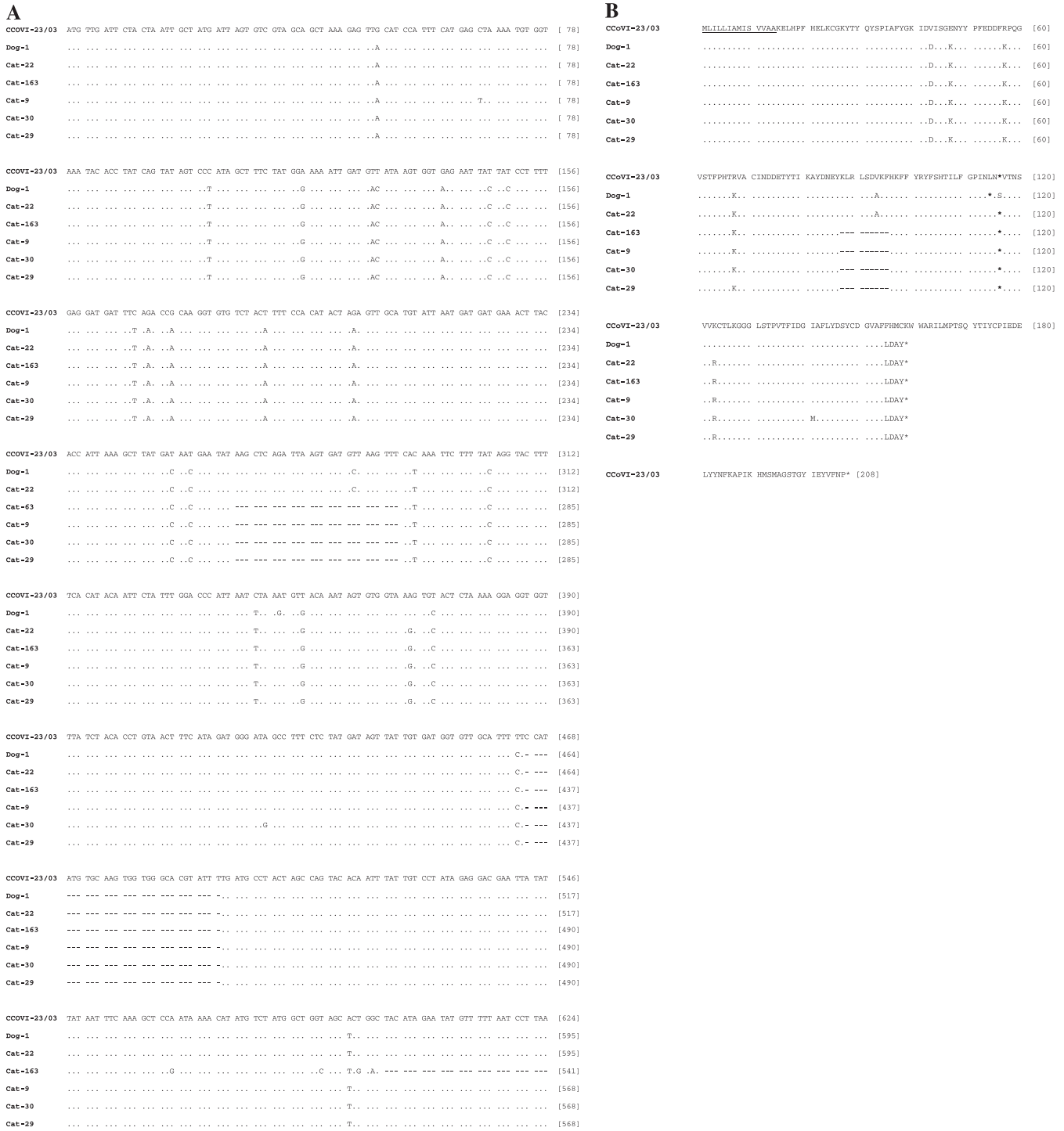


Fig. 3. Alignment of nucleotide (A) and amino acid (B) ORF3 sequences in comparison with the sequence from the reference CCoV-I 23/03 strain. A conserved potential signal peptide is underlined, a potential N-glycosylation site is indicated by an asterisk in bold (B).

the potential N-glycosylation site, described in intact gp3 proteins (Fig. 3B).

4. Discussion

The data of molecular biology of FCoV and CCoV have rapidly accumulated since the early 2000s. FCoV and CCoV strains are classified into 2 main genotypes sharing close relationships. In particular, the S protein from FCoV-II originates from CCoV-II and the S

protein of FCoV-I and CCoV-I share 81% identity, which could potentially lead to interspecific transmission of coronaviruses because S mediates cell entry. By sequence analysis of fragments from the M and S genes, Benetka et al. suggested that interspecies transmission of CoVs occurred in a shelter where cats and dogs were in contact. They indeed detected atypical FCoV strains related to CCoV-I (Benetka et al., 2006). However, since the ORF3 gene had not been described at this time, it was impossible to confirm that these atypical strains belonged to the CCoV-I genotype.

The present study was conducted on cats from private owners with the aim to investigate (i) the possible influence of the presence of dogs to the prevalence of cats coronavirus infection and (ii) the genetic characterisation of FCoV strains and CCoV strains from dogs living with CoV infected cats. We collected 88 faecal samples from healthy cats and 11 ascitic fluid from cats with clinical symptom of FIP. Among them, 19 were living with at least one dog in the same household but the percentages of infected cats and non-infected cats in close contact with a dog were not statistically different (Table 1).

Phylogenetic analysis was performed on 15 coronavirus strains collected from healthy cats, 11 FIP cats and one dog by first sequencing fragments from N and S genes (Figs. 1,2). Most of the FCoV strains were identified as FCoV-I with the N and S genes clustering with reference FCoV-I strains. Surprisingly, the N sequences from six strains recovered from Cats -9, -22, -29, -30, -163 and 179 clustered within the CCoV-I genotype whereas the spike gene of 4 of them (Cats -22, -29, -30 and 163) segregated within the FCoV-I genotype. One significant element is the presence of the accessory ORF3 gene in these atypical FCoV strains, as this gene was described to be peculiar to CCoV-I strains (Lorusso et al., 2008). Lorusso et al. hypothesised that, CCoV-I may have acquired the ORF3 gene after the divergence of FCoV-I and CCoV-I from their common ancestor, or, alternatively that FCoV-I may have lost the ORF3 gene present in the common parental virus. However, the description of atypical FCoV-I strains harbouring ORF3 gene led to the hypothesis that the ORF3 gene also persisted in some FCoV-I or that these strains may originate from a recombination between FCoV-I and CCoV-I strains. In the present study, we identified one couple of cat and dog (Cat-22 and Dog-1) harbouring coronavirus and living together. Notably, Dog-1 was infected by a CCoV-I strain and Cat-22 by an atypical FCoV-I strain comprising an ORF3 gene. Moreover, strains infecting these animals shared 99% of nucleotide identity within the N gene, the ORF3 genes exhibited the same 29-nt deletion which yielded a stop codon but the S genes significantly differed. It is therefore tempting to speculate that the strain detected in Cat-22 originated from recombination with the CCoV-I strain from the Dog-1. However, without extensive sequence analysis of the full-length genome from some atypical FCoV-I harbouring an ORF3 gene, it would be impossible to clarify whether they originate or not from recombination with CCoV-I and to identify the putative recombination sites.

Curiously, the ORF3 genes amplified herein were found to have one or two deletions, never described. All had the same 29-nt deletion which yielded a stop codon and four of the six obtained ORF3 genes shared another 27-nt in frame deletion (Fig 3). So far all sequenced ORF3 genes originated from Italy and all of 623 bp in length (Lorusso et al., 2008). More information regarding the variability of this gene will be necessary to determine if these deletions are essentially restrained to cats viruses or if they are also spread among the canine strains.

In this study, all cats, except Cat-163 were living in the same French geographic region, moreover Cat-22/Dog-1 and Cat-29/Cat-30 were in close contact, which could explain the high degree of sequence homology between the ORF3 sequences amplified herein. Only Cat-163 originated from Romania and its ORF3 sequence diverged from the others, with a shortening after the stop codon introduced by the 29-nt deletion (Fig. 3A).

ORF3 belongs to the group of accessory genes which are characteristic of each genus of the *Coronaviridae* family. The functions of accessory proteins are often unknown but they are regularly implied in tropism switch or in adaptation of a viral strain to a new host species. Thus, adaptation of SARS-CoV from the bat to human was accompanied by several mutations in different parts of the genome including a characteristic 29-nt deletion within the accessory ORF8 gene (Chinese SMEC, 2004; Oostra et al., 2007). The

function of the gp3 protein encoded by the intact ORF3 gene is still unknown. Its biochemical properties, glycosylation and molecular weight have been studied by *in vitro* translation assays (Lorusso et al., 2008). Gp3 is a 28 kDa N-glycosylated protein with a cleavable N-terminal signal, indicating that it may be a secretory protein. According to the predictive computer analysis, the truncated gp3 derived from the deleted ORF3 genes retain the same glycosylation site and the signal peptide. More studies on the biological properties of gp3 and its putative truncated counterparts described herein will be necessary to understand their role.

5. Conclusion

Finally, our data demonstrate for the first time the circulation of atypical feline coronaviruses harbouring a truncated form of the ORF3 gene, whereas it was so far described as peculiar to CCoV-I strains. Clarifications must be made upon whether these strains are widespread amongst the cat population and full-length genome sequencing will be required to deepen the phylogenetic analysis. Given the dramatic consequences of SARS-CoV infection and more recently the new MERS-CoV, greater understanding of the molecular processes leading to the emergence of coronaviruses remains crucial (Zaki et al., 2012). In this context the surveillance of animal coronaviruses remains important not only because animal coronaviruses are often responsible of major veterinary diseases but also because the knowledge accumulated have a substantial contribution to the understanding of the genetic evolution and pathobiology of coronaviruses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.meegid.2013.09.024>.

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