# Sequence analysis of the nucleocapsid gene of feline coronaviruses circulating in Italy

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**SUMMARY**

Molecular analysis of the N genes of feline coronaviruses (FCoV) strains detected in naturally infected cats were carried out to investigate the genetic diversity among these viruses. Phylogeny showed a general clustering trend on the basis of geographic origin rather than on virulence characteristics. The analysis of the pattern of nucleotide substitutions disclosed "hot spots" sites which may represent immunological domains. In conclusion, our results demonstrate that the N gene does not carry mutations associated with the pathotypical switch FECV→FIPV. During persistent infection, the individual qualitative immune response might address the accumulations of mutations in the N gene and the development of FIP.

*KEY WORDS:* Feline infectious peritonitis, Feline coronaviruses, N protein, Phylogenetic analysis, Italy

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Feline coronaviruses (FCoVs) are enveloped, positive-stranded RNA viruses belonging to family *Coronaviridae* within the order *Nidovirales* (Lai *et al.*, 2007).

FCoV infection is extremely common in cats worldwide. Most natural infections are subclinical and result in self-limiting gastrointestinal disease: in these cases, the causative agent is known as feline enteric coronavirus (FECV biotype) (Pedersen, 1995; Addie *et al*., 1995). Only a small percentage of infected cats develop the classical symptoms of feline infectious peritonitis (FIP), a fatal immune-mediated disease which is caused by a virulent variant of FCoVs, the FIP virus (FIPV biotype).

Several theories have been proposed to explain the mechanisms leading a cat infected by FCoV to develop a fatal systemic effusive form (FIP) rather than a subclinical enteric disease:

1. The "internal mutation" theory asserts that the virulent pathotype FIPV evolves from the avirulent FECV by genetic mutation in individual infected cats (Vennema *et al.*, 1998; Pedersen, 2009). The mutation responsible for the FIPV biotype is consistently found on the 3c gene, which encodes a small protein of unknown function (Pedersen *et al*. 2009), but it is certainly not the only one involved in the pathotypic switch (Chang *et al*. 2010).
2. Brown *et al.* (2009) calls into question the *in vivo* mutation hypothesis, since they observed monophyletic clustering of strains correlating with disease phenotype in membrane and 7b genes, consistent with distinctive circulating virulent and avirulent strains in natural populations.
3. Finally, several studies have implied that whether a cat develops FIP after FECV exposure is determined by how the host responds to the virus and it is independent from genetic differences in biotype. Host and environmental factors play a crucial role in whether or not FIP is clinically manifested (Kipar *et al*., 2006; Addie *et al*., 2004).

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All coronaviruses contain at least three main structural proteins: spike (S), membrane (M) and nucleocapsid (N).
The nucleocapsid (N) protein is one of the most abundant viral proteins produced throughout viral infection and plays a role in viral replication, assembly and immunity (Lai *et al.*, 2007).

The major goal of this study was to investigate the molecular diversity of the N protein in FCoV strains collected from clinically healthy carrier and diseased cats from different geographic areas. Our choice to analyze the N gene was due to the fact that the N protein is a relatively conservative and highly immunogenic protein among

TABLE 1 - *Details of viral strains analyzed.*

| *Group* | *Viral strains*[a] | *GenBank accession number* | *Specimen source*[b] | *Clinical status*[c] |
|---|---|---|---|---|
| Shelter A | TN/352N/00 | / | RS | H |
| | TN/352C/00 | GU017124 | RS | H |
| | TN/352S/00 | / | RS | H |
| | TN/361/00 | GU017122 | RS | H |
| | TN/366/00 | GU017123 | RS | H |
| | TN/368/00 | / | RS | H |
| | TN/369/00 | GU017126 | RS | H |
| | TN/376/00 | GU017125 | RS | H |
| | TN/419/00 | GU017118 | B | FIP-DF |
| | TN/420/00 | GU017119 | L | FIP-EF |
| Shelter B | PC/Aron/05 | GU017105 | RS | H |
| | PC/Babette/05 | GU017111 | RS | H |
| | PC/Gigia/05 | GU017106 | RS | H |
| | PC/Serena/05 | GU017112 | RS | H |
| | PC/S739/05 | GU017107 | RS | H |
| | PC/S1196/05 | GU017108 | RS | H |
| | PC/Fedro/05 | GU017109 | RS | H |
| | PC/Seven/05 | GU017110 | PF | FIP-EF |
| | PC/Puffo/05 | GU017113 | RS | H |
| | PC/Luna/05 | GU017115 | RS | H |
| | PC/Falkon/05 | GU017117 | RS | H |
| | PC/Bimba/05 | GU017121 | RS | H |
| | PC/Alan/05 | GU017102 | RS | H |
| | PC/Mattia/05 | GU017116 | RS | H |
| | PC/M314/06 | GU017104 | RS | H |
| | PC/M477/06 | GU017103 | RS | H |
| | PC/Susanna/06 | GU017114 | RS | H |
| Cat Breeding C | MI/cat1/04 | GU017099 | RS | H |
| | MI/cat2/04 | GU017095 | RS | H |
| | MI/cat3/04 | GU017093 | RS | H |
| | MI/cat4/04 | GU017100 | RS | H |
| | MI/cat5/04 | GU017094 | RS | H |
| | MI/cat6/04 | GU017091 | RS | H |
| | MI/cat7/04 | GU017092 | RS | H |
| | MI/cat8/04 | GU017120 | RS | H |
| | MI/cat9/04 | GU017097 | RS | H |
| | MI/cat10/04 | GU017098 | RS | H |
| | MI/203/05 | GU017096 | LN | FIP-EF |
| | MI/216/05 | GU017101 | LN | FIP-EF |

[a]Province of strain origin (TN=Trento; PC=Piacenza; MI=Milan)/Sample identification/Year. [b]RS=Rectal swab, B=Brain, L=Liver, PF=Peritoneal fluid, LN=Lymph nodes. [c]Clinical presentation associated with each strain; FIP confirmed by RT-PCR one tube positive results from clinical specimens and necroscopy (Gut et al., 1999). H = Healthy; FIP-DF = FIP-Dry form; FIP-EF = FIP-Effusive form.

coronaviruses which can provoke a protective immune response and is also an important diagnostic marker for coronavirus disease (Leung *et al*., 2004). Furthermore the N protein (despite not being a surface protein) still remains a relevant factor in the immunopathogenicity of FIP. Several studies suggested a role of the N protein in stimulating cell-mediated immunity against feline coronaviruses, the only immunity which appears to play a protective role (Wasmoen *et al*., 1995; Hohdatsu *et al*., 2003).

FCoV strains were obtained from naturally infected colonies of domestic cats, designated A, B and C. Most viruses have already been included in previous studies to determine the prevalence of FCoVs, identify carriers and shedders, and investigate the quasispecies nature of FCoVs (Paltrinieri *et al*., 2007; Battilani *et al*., 2003). Five viruses were detected in extra-intestinal organs of subjects showing clinical signs of FIP and they were referred to as virulent strains or FIPV-like; the remaining strains were detected in faeces of healthy cats and were referred to as avirulent strains or FECV-like (Dye *et al*., 2007). The characteristics of the samples examined are summarized in Table 1.

Viral RNA was extracted from clinical samples and nested RT-PCR was performed to amplify the entire N gene as previously described (Vennema *et al*., 1998; Battilani *et al*., 2006). Amplified products were purified and sequenced.

Nucleotide sequence alignments of the N gene were carried out in CLUSTAL W web interface and the nucleotide alignments for the codon positions were subsequently corrected by DAMBE software version 4.1.19 (Larkin *et al*., 2007; Xia *et al*., 2001). Final alignments were manually edited and translated into amino acid sequences; the degree of similarity among the sequences at both the nucleotide and the amino acid levels was determined using the BIOEDIT sequence alignment editor version 7.0.9 (Hall, 1999). The alignments are available from the authors on request.

Phylogenetic analysis was carried out using the maximum likelihood (ML) approach. Maximum likelihood parameters were estimated using TREEPUZZLE software version 5.2 (Schmidt *et al*., 2002). Neighbor-joining (NJ) trees were constructed with PHYLIP software package version 3.67 (Felsenstein, 1989), using previously estimated maximum likelihood parameters and the HKY85 evolution model for nucleotide sequences. Protein distance calculations were based on the Jones-Taylor-Thornton protein weight matrix.

The reliability of the phylogenetic trees obtained was evaluated by running 1000 replicates using the bootstrap test (Felsenstein, 1985).

In order to identify the mechanism underlying the diversification of strains within the individual groups, evidence of positive selection was sought using a codon-based approach as implemented in Datamonkey (Pond *et al*., 2005). All the methods implemented in the Datamonkey web interface were run, and comparative analysis integration was carried out: a single likelihood ancestor counting (SLAC) analysis was performed using a P value of 0.1; fixed effects likelihood (FEL) and internal fixed effects likelihood (IFEL) were run using P=0.25, and random effects likelihood (REL) was run with a Bayes Factor of 50.

The entire sequence of the N gene ranged from 1122-1137 base pairs (bps). In Shelter A, comparison of the N gene sequences showed 100% nucleotide identity between avirulent strains TN/352C/00, TN/352S/00 and TN/352N/0; TN/366/00 and TN/368/00. The other strains showed a sequence similarity which varied from 89.7 to 99.9%. The amino acid sequences revealed are identical in the TN-366, TN-368 and TN-376 strains while the remaining viral strains differed in identity by 90.7 to 99.7%.

In Shelter B, the percentage of nucleotide identity ranged from 90.9 to 99.4%; in the amino acid sequences revealed, similarity varied from 91.7 to 99.4%.

In Cat Breeding C, N gene alignment showed that the percentage of nucleotide identity ranged from 90.6 to 99.6%. Values of amino acid identity ranged from 91.2 to 99.4%.

However, within each colony, the nucleotide divergence among strains was much higher than those previously reported for epidemiologically related isolates, ranging up to 10.2, 8.5 and 9% within colonies A, B and C, respectively. These results suggest that endemically infected colonies provide a favourable environment for the generation of FCoVs genetic diversity.

Global alignment of nucleotide and translated amino acid sequences has shown that the major part of mutation insertions and deletions (InDel)

are located in the protein region, ranging from residue 180 to residue 210.
The mutations seem to be casual and they are not correlated to the virulence characteristics of the strains. Despite the elevated variability between the sequences, distinct mutations capable of distinguishing between virulent and avirulent strains were not observed.
Datamonkey analysis was carried out to examine the pattern of nucleotide substitutions and to determine whether particular sites in the N gene were subjected to positive selection. The SLAC method does not detect sites subjected to negative or positive selection, whereas FEL, IFEL and REL analysis demonstrated different amino acids subjected to positive selection. We considered as "under selection" only those sites recognized by at least two methods; in Shelter A, residues 181, 187 and 220, in Shelter B, amino acids 30, 132, 136, 182, 186, 203 and 337 and in Cat Breeding C, amino acids 30, 39, 225 and 245. These sites subjected to positive selection could represent antigenic immunodominant sites and it is therefore likely that pressure of the host immune systems leads to repeated amino acid replacements.
Phylogenetic analysis demonstrated a general clustering trend on the basis of geographic origin rather than on virulence characteristics with some exceptions.
The phylogenetic trees showed a close relationship for Shelter B and Cat Breeding C with distinct clusters for each group, but they also showed a strict correlation between some avirulent strains detected in chronic carrier cats and virulent strains, despite their different geographic origins (Figure 1).
Previous surveys carried out on Cat Breeding C, to investigate faecal shedding of FCoVs in clinically healthy cats, disclosed several chronic carrier cats (cats 1, 4, 7, 8) with persistent infection (Paltrinieri *et al.*, 2007). Sequences detected in these persistent shedders had the tendency of clustering together with virulent strains; strains MI/Cat1/04 and MI/Cat4/04 formed a separate cluster with the virulent strain MI/216/05, and MI/cat8/04 constituted a distinct subgroup with the virulent strains of Shelter A; both subgroups were supported by a bootstrap value of 100%. Probably, during persistent infection viral replication leads to the accumulation of nucleotide substitutions in the viral genome responsible for increasing virulence, but host factors may play an important role in blocking the development of FIP.
Furthermore, the sequences from Shelter A have the tendency to cluster on the basis of virulence characteristics rather than geographic origin. In fact, in our previous study, we carried out computational antigenic analysis on N sequences of strains from Shelter A, with the aim of detecting antigenic sites on N protein. Antigenic analysis carried out using several algorithms demonstrated a substantial difference between virulent and avirulent strains in the mapping of antigenic sites. all avirulent strains from healthy cats showed ad-

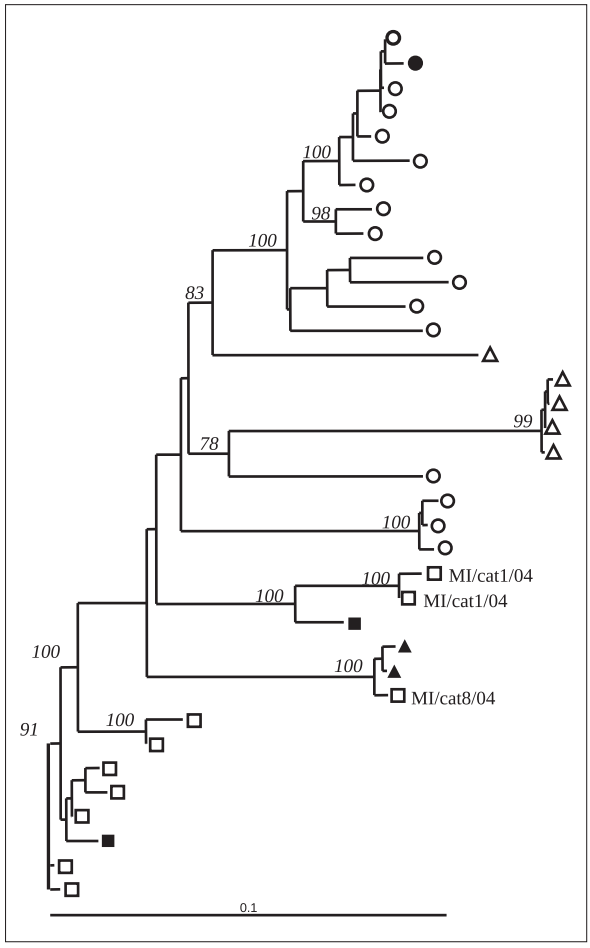


FIGURE 1 - *Dendrogram obtained with all N protein sequences; the clusters which define the relationships between FCoVs strains are shown. Significant bootstrap values (>80%) and the clustering of viruses analysed into groups are indicated. Legend:*
*Shelter A: △ Avirulent strains - ▲ Virulent strains*
*Shelter B: Avirulent strains - Virulent strains*
*Cat Breeding C: □ Avirulent strains - ■ Virulent strains*

ditional putative antigenic sites which are not present in the virulent strains. On the basis of these results, we speculated that virulent strains could elude immune surveillance, removing the immunodominant motifs from the N protein sequences (Battilani *et al.,* 2006). No significant differences were detected when reference strains were included in the phylogenetic analysis.

In conclusion, the high genetic correlation found between virulent/avirulent strains and between geographically distant sequences, demonstrates that the N gene does not carry mutations associated with the pathotypic switch. Previous studies have shown that the CoV N protein is the most "immuno-dominant" antigen in the host immune response (Tang *et al.*, 2005).

The presence of several sites under positive selection in the analyzed sequences, which might be immunodominant antigenic sites, support the potential antigenic role of N protein. During persistent FCoV infection, the individual qualitative immune response might addresses the accumulations of mutations in N gene and the development of FIP.

Further investigations will clarify the potential immunogenic role of N protein. Any information generated from the analysis of this protein will definitely boraden our understanding of the biology of FCoV infection allowing the design of improved preventive tools.

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