Variation of the sequence in the gene encoding for transmembrane protein M of canine coronavirus (CCV)

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A nucleotide variability in the sequence of the gene encoding for the transmembrane protein M of canine coronavirus (CCV) is described. A total of 177 faecal samples from pups with enteritis were analysed by a PCR and n-PCR specific for CCV. Four samples, collected from a dog presenting a long-duration shedding of CCV, and a sample from another diarrhoeic dog, were found positive by PCR but negative by n-PCR. Sequence analysis of the samples revealed silent nucleotide substitutions in the binding site of the internal primer used for the n-PCR. Moreover, the nucleotide substitutions occurring over the whole fragment of the five samples analysed were similar.

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INTRODUCTION

Canine coronavirus (CCV), a member of the *Coronaviridae* family, was first isolated in 1971 during an epizootic in Germany from a case of canine enteritis.[1] CCV is serologically and genetically related to other coronaviruses: feline infectious peritonitis virus (FIPV), feline enteric coronavirus (FeCV), transmissible gastroenteritis virus (TGEV) and porcine respiratory coronavirus (PRCV).[2–4]

In naturally occurring infections, pups between six and 12 weeks old appear to be more susceptible to disease[5–7] and this may be related to the decline of maternal antibodies.[8] In young pups CCV appears to replicate primarily in enterocytes on the villus tips of the small intestine, but also in the epithelium of the large intestine,[7] and was excreted in the faeces.[1,9,10] CCV may cause diarrhoea, vomiting and dehydration, loss of appetite, lethargy, and occasionally sudden death of younger pups.

The isolation of CCV from infected dogs is often difficult and, frequently, coronavirus-like particles seen by electron microscopy (EM) in stools have been difficult to propagate. These problems in isolating CCV still hinder studies on their epidemiology and pathogenesis.

Recently a PCR and n-PCR have been developed for the diagnosis of CCV to overcame the difficulties in virus isolation *in vitro*.[11,12] Our original PCR and n-PCR assays are based on a hemi-nested strategy. The pair of primers used for the PCR were chosen in a well-conserved region of the M gene of CCV and are able to amplify also FIPV and TGEV strains. The primer pair used in the n-PCR was able to amplify only CCV and TGEV strains. By using this heminested strategy we were able to detect CCV RNA with a

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higher sensitivity (approximately of a 100-fold dilution) than PCR.

During an epidemiological survey for CCV using n-PCR on faecal samples of dogs with enteritis, some samples resulted positive to CCV by PCR, but, unexpectedly, negative by n-PCR. With the aim of investigating these occasional findings, the sequence of the samples displaying unusual response to the n-PCR assay was determined and analysed.

MATERIALS AND METHODS

Faecal samples

A total of 177 faecal samples of dogs were examined. The samples were grouped as follows: A: 71 samples were collected during a period of about 12 months and stored at −20°C; B: 15 samples were fresh rectal swabs collected from pups with enteritis; C: 91 faecal samples were collected, approximately every three days, from a dog recovered from CCV enteritis and kept under observation for a period of nine months.

Virological examinations

Only the rectal swabs of group B and C ($n=106$) were subjected to viral isolation. The samples were homogenized in Dulbecco-Minimal Essential Medium (D-MEM) and clarified by centrifugation at 4000 *g* for 20 min at 4°C. The supernatant of each sample was treated with antibiotics (5000 IU/ml penicillin, 2500 µg/ml streptomycin, 10 µg/ml amphotericin) for 30 min at 37°C, inoculated onto partially confluent monolayer of canine cell culture (A-72), and then incubated at 37°C in a 5% (v/v) CO_2 incubator. Each sample was considered negative for CCV if, after three passages, no cytopathic effect (cpe) was observed. In the presence of cpe, the isolate was identified by indirect immunofluorescence assay (IFA) using monoclonal antibodies (MoAbs) to coronaviruses.

PCR and nested-PCR (n-PCR)

The faecal samples were tested for CCV by PCR and n-PCR as previously reported.[11] Briefly, specimens were diluted 1 : 100 in phosphate-buffered saline (PBS) and homogenised by vigorous vortexing. Insoluble components were removed by centrifugation for 5 min at 8000 *g*, and genomic RNA was extracted using the Rneasy Total RNA kit (Qiagen GmbH,

Table 1. Primers used for the PCR and n-PCR assays

Primer	Sequence 5′→3′	Sense	Position
CCV1	TCCAGATATGTAATGTTCGG	+	337–356
CCV2	TCTGTTGAGTAATCACCAGCT	−	726–746
CCV3	GGTGTCACTCTAACATTGCT	+	535–555

Germany) from 1 ml of the supernatant fraction of each sample.

The target sequence for amplification is a segment (nucleotides 337–746) of the gene encoding for transmembrane protein M of CCV.

The reverse transcriptase (RT) was performed in a total reaction volume of 20 µl containing 1 × PCR buffer (KCl 50 mM; Tris-HCl 10 mM; pH 8·3), $MgCl_2$ 5 mM, 1 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), Rnase inhibitor 1 U, RT 2·5 U, 50 pmol primer antisense CCV2. The cDNA syntheses was carried out at 37°C for 30 min, followed by a denaturation step at 94°C for 5 min.

The PCR reaction, in a total volume of 100 µl containing 1 × PCR buffer, $MgCl_2$ 2 mM, 0·25 mM of each deoxynucleotide, *Amplitaq* Gold DNA polymerase 1·25 U, 50 pmol primer sense CCV1, was carried out for 34 cycles in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, USA), with the following reaction parameters: template denaturation 94°C for 1 min, primer annealing 55°C for 1 min, and extension at 72°C for 3 min. A single final extension step was done at 72°C for 10 min to complete the amplification reaction.

For the n-PCR, a 20 µl aliquot of the 1 : 100 dilution of the first amplicon was subjected to a second round of amplification using the CCV2 and CCV3 primers and the same PCR cycling procedures.

Amplified products were electrophoresed on 2% (w/v) agarose gel at 50 V for about 120 min and stained with the ethidium bromide. The sequence and position of the primers are shown in Table 1.

Sequence analysis

The samples examined were from group A (A-32/99) and from group C (C-5/4, C-10/4, C-11/7 and C-15/7). The samples from group C were collected, respectively, two months (C-5/4 and C-10/4) and five months (C-11/7 and C-15/7) after the presumed primary infection of the dog. All these samples were negative by the n-PCR. Sequence analysis was also performed on the PCR amplicon of sample C-22/2, collected from the pup at the onset of enteritis and resulted, as expected, positive by n-PCR.

Table 2. Results of the PCR and n-PCR assay for CCV carried out on faecal samples from dogs

Group	Samples	PCR positive	n-PCR positive
A	71	14	30
B	15	15	15
C	91	9	26

The RT-PCR amplicons, obtained with the CCV1-CCV2 primer pair, were purified on Ultrafree-DA Columns (Amicon, Millipore) and then sequenced directly with ABI-PRISM 377 (Perkin Elmer, Applied Biosystem Instruments). Each sample was sequenced twice. Sequence analysis was performed with NCBI's and EMBL's analysis tools. The alignment of the sequences was performed with CLUSTAL W.[13] For alignment and comparative analysis, published sequence of the M gene of CCV strain Insavc (accession number D 13096) was used.

RESULTS

Four CCV strains out of the 106 faecal samples examined from groups B and C, were isolated on A-72 cells. Two isolates were made from group B, while two isolates were made from the pup kept under observation (group C), from samples collected three and five days after the onset of enteritis. The isolates induced the typical lytic cpe of coronavirus after three days of incubation and the A-72 cells resulted positive by the IFI test using MoAbs.

In Table 2 are reported the results of PCR and n-PCR analysis for CCV. In group A, 14/71 samples resulted positive by the PCR assay and 30/71 were positive by the n-PCR assay. All the 15 fresh samples from pups with enteritis (group B) resulted positive both by the PCR and n-PCR assays. Twenty-two samples collected from the pup kept under observation (group C) resulted positive by PCR, and 28 samples by n-PCR. Interestingly, shedding of CCV occurred intermittently over a period of nine months.

Five rectal swabs, one (A-32/99) from group A and four (C-5/4, C-10/4, C-11/7 and C-15/7) from the pup kept under observation (group C) resulted positive by PCR but negative by n-PCR. Sequence analysis of these five samples revealed repetitive nucleotide substitutions in the binding site of the internal primer CCV3, though these nucleotide variations do not determine amino acid changes (data not shown).

The sequences of the five PCR amplicons resulted highly similar to each other. Several mutations observed in sample A-32/99 occurred in the same positions as samples C-5/4, C-10/4, C-11/7 and C-15/7, which were collected from the dog with long shedding of CCV. All the five samples were different from CCV reference strain Insavc, and, interestingly, from sample C-22/2, which was collected at the onset of enteritis from the dog with long viral shedding. There were a few differences between samples C-5/4 and C-10/4 and between samples C-11/7 and C-15/7, collected, respectively, two and five months after the onset of the enteritis. Moreover, samples C-5/4 and C-10/4 were slightly different from samples C-11/7 and C-15/7 (Figure 1).

DISCUSSION

The M glycoprotein of coronaviruses is a transmembrane molecule deeply embedded in the viral envelope so that only a small region is exposed on the outer surface of the lipid bylayer. Its function is essential for coronaviruses budding from rough endoplasmic reticulum (RER) and Golgi membranes. Antibodies to M glycoprotein can neutralize viral infectivity, but only in the presence of complement.[14,15]

At the moment, the significance of the variations observed in the gene of the M glycoprotein of CCV is not completely clear. The leader-primed replication and transcription of coronavirus RNA result in a high frequency of recombination and also of mutation because most RNA polymerases would probably introduce one or more mutations during every round of replication of the long genome of coronaviruses, generating, as consequence, a great deal of genetic variations in the progeny of coronaviruses from one infected individual.[16] A high degree of genetic variation in FeCV RNA has been already revealed by sequence analysis of the 7b and S genes and by denaturing gradient gel electrophoresis of PCR amplicons of the S and N genes, suggesting that each feline coronavirus exists as a quasispecies.[17–19] The findings of our study, though based on a limited number of samples and on a short fragment of CCV RNA, suggest that a high degree of variation in the genome of CCV also exists.

In our study, five samples were found to be positive by PCR, but, unexpectedly, negative by n-PCR, though the n-PCR is more sensitive than the PCR assay.[11] All these samples exhibited similar variations in the binding site of the internal primer CCV3 used in the n-PCR, with one of the nucleotide variations occurring at the very 3′ terminus of the primer binding site, which is important for the stability of primer annealing. In regard to sample A-32/99, there are only three substitutions in this region and they are not in the 3′ terminus. In this case, it may be supposed that

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C-22/2  6766  CAATTGTTACATTTATACTTTGGATTATGTATTTTATTAGATCCATTCAGTTATACAGAA
C-5/4         ..G..........GC....................G.C.....T......C.........
C-10/4        ..G..........GC....................G.C.....T......C.........
C-11/7        ..G..A.......GC....................G.A.....T......C....T....
C-15/7        ..G..A.......GC....................G.A.....T......C....T....
A-32/99       ..G..A.......GC....................G.A.....T......C....T....
Insavc        ...C..............................G........................


C-22/2        GGACTAAGTCTTGGTGGTCTTTCAACCCTGAAACTAACGCAATTCTTTGCGTTAGTGCAT
C-5/4         ..............................T..........T..........T.........C
C-10/4        ..............................T..........T..........T.........C
C-11/7        ..............................T..........T..........T.....C...C
C-15/7        ..............................T..........T..........T.....C...C
A-32/99       ..............................T.....G....T.......A...........GC
Insavc        .................................G....................G.


C-22/2        TAGGAAGAAGCTACGTGCTTCCTCTTGAAGGTGTGCCAACT┌GGTGTCACTCTAACATTGC┐
C-5/4         .T..........T..................ACA......│.....T.....T..T..A.│
C-10/4        .T..........T..................ACA......│.....T.....T..T..A.│
C-11/7        .T.............................ACA......│.....T..C..T......T│
C-15/7        .T.............................ACA......│.....T..C..T......T│
A-32/99       .T..........T..................ACA......│.....T.....T..T....│
Insavc        ............T...........................└...................┘


C-22/2        ┌T┐CTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACATCGACAATT
C-5/4         │.│A.....A...C.T...........A..T.....G..T.....A..........GC...
C-10/4        │.│A.....A...C.T...........A..T.....G..T.....A..........GC...
C-11/7        │.│A.....A...C.T..............T.....G..T.....A..........GC...
C-15/7        │.│A.....A...C.T..............T.....G..T.....A..........GC...
A-32/99       │.│A.....A...C.T...........A..T.....G..T.....A..........GC...
Insavc        └.┘T...........G...............................................


C-22/2        TACCAAAATACGTAATGGTTGCATTACCTAGCAGGACCATTGTCTACACACTTGTTGGCA
C-5/4         ...............................T..A..T....................A.
C-10/4        ...............................T..A..T....................A.
C-11/7        .................C.............T..A..T....................A.
C-15/7        .................C.............T..A..T....................A.
A-32/99       .................C......T....T..A..T....................A.
Insavc        ..........T.....GC..............GT...A.....A................


C-22/2        AGAAATTGAAAGCAAGTAGTGCGACAGGATGGGCTTACTATGTAAAATCTAAAGCTGGTG
C-5/4         ..C....................A....................................
C-10/4        ..C....................A....................................
C-11/7        ..C....................A....................................
C-15/7        ..C....................A....................................
A-32/99       ..C....................A....................................
Insavc        .......................A.......................G............


C-22/2  7125  ATTAC
C-5/4         .....
C-10/4        .....
C-11/7        .....
C-15/7        .....
A-32/99       .....
Insavc        .....
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Fig. 1. Alignment of the M protein nucleotide sequences of CCVs from faecal samples of pups. The nucleotide position is referred to the reference strain Insavc. The binding site of primer CCV3 is boxed. Identities are indicated by a dot.

these variations affects partially the efficiency of the n-PCR amplification, with the production of not detectable amount of cDNA, under our PCR conditions.

Four samples (C-5/4, C-10/4, C-11/7 and C-15/7) were collected from the dog, in which viral shedding was detected intermittently for nine months. These samples are quite different from a sample (C-22/2) collected in the same dog at the beginning of the viral shedding. A variability in the S gene of geographically distinct isolates of CCV has been already reported.[20] Nevertheless, we observed a genetic variation in the M gene of samples collected from the same animal. Therefore the variation observed does not reflect a geographical differentiation. Whether these nucleotide variations represent a mechanism allowing for the persistence of CCV in the host may not be hypothesized without further studies, especially on the immune response of dogs against the surface viral proteins M and S.

REFERENCES

1. Binn, L. N., Lazar, E. C., Keenan, K. P., Huxsoll, D. L., Marchwicki, B. S. & Strano, A. J. (1974). Recovery and characterization of a coronavirus from military dogs with diarrhoea. *Proceedings of the 78th Meeting of the US Animal Health Association*, 359–66.
2. Horsburgh, B. C., Brierley, I. & Brown, T. D. K. (1992). Analysis of a 9·6 kb sequence from the 3′ end of canine coronavirus genomic RNA. *Journal of General Virology* **73**, 2849–62.
3. Sanchez, C. M., Jimenez, G., Laviada, M. D., Correa, I., Sune, C., Builldo, M. J. *et al.* (1990). Antigenic homology among coronaviruses related to transmissible gastroenteritis virus. *Virology* **174**, 410–7.
4. Wesseling, J. G., Vennema, H., Godeke, G. J., Horzinek, M. C. & Rottier, P. J. M. (1994). Nucleotide sequence and expression of the spike (S) gene of canine coronavirus with the S protein of feline and porcine coronaviruses. *Journal of General Virology* **75**, 1789–94.
5. Carmichael, L. E. & Binn, L. N. (1981). New enteric viruses in the dog. *Advances in Veterinary Science and Comparative Medicine* **25**, 1–37.
6. Cartwright, S. & Lucas, M. (1972). Vomiting and diarrhoea in dogs. *Veterinary Record* **91**, 571–2.
7. Vandenberghe, J., Ducatelle, R., Debouck, P. & Hoorens, J. (1980). Coronavirus infection in a litter of pups. *Veterinary Quarterly* **2**, 136–41.
8. Tennant, B. J., Gaskell, R. M., Kelly, D. F., Carter, S. C. & Gaskell, C. J. (1991). Canine coronavirus infection in dog following oronasal inoculation. *Research in Veterinary Science* **51**, 11–8.
9. Keenan, K. P., Jervis, H. R., Marchwicki, R. H. & Binn, L. N. (1976). Intestinal infection of neonatal dogs with canine coronavirus 1–71: studies by virologic, histologic, histochemical and immunofluorescent techniques. *American Journal of Veterinary Research* **37**, 247–56.
10. Takeuchi, A., Binn, L. N., Jervis, H. R., Keenan, K. P., Hildebrandt, P. K., Valas, R. B. *et al.* (1976). Electron microscope study of experimental enteric infections in neonatal dogs with a canine coronavirus. *Laboratory Investigation* **34**, 539–49.
11. Pratelli, A., Tempesta, M., Greco, G., Martella, V. & Buonavoglia, C. (1999). Development of a nested PCR assay for the detection of canine coronavirus. *Journal of Virological Methods* **80**, 11–5.
12. Pratelli, A., Buonavoglia, D., Martella, V., Tempesta, M., Lavazza, A. & Buonavoglia, C. (2000). Diagnosis of canine coronavirus infection using n-PCR. *Journal of Virological Methods* **84**, 91–4.
13. Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–80.
14. Collins, A. R., Knobler, R. L., Powell, H. & Buchmeier, M. J. (1982). Monoclonal antibodies tp murine hepatitis virus-4 (strain JHM) define the viral glicoprotein responsible for attachment and cell-cell fusion. *Virology* **119**, 358–71.
15. Risco, C., Anton, I. M., Sune, C., Pedregosa, A. M., Martin-Alonso, J. M., Parra, F. *et al.* (1995). Membrane protein molecules of transmissible gastroenteritis coronavirus also expose the carboxy-terminal region on the external surface of the virion. *Journal of Virology* **68**, 5269–77.
16. Kathryn, V. H. (1991). Coronaviridae and their replication. In *Fundamental Virology* (B. N. Fields, D. M. Knipe, eds.) Pp. 471–86. New York: Raven Press Ltd.
17. Gunn-Moore, D. A., Gunn-Moore, F. J., Gruffydd-Jones, T. J. & Harbour, D. A. (1999). Detection of FCoV quasispecies using denaturing gradient gel electrophoresis. *Veterinary Microbiology* **69**, 127–30.
18. Herrewegh, A. A. P. M., Mähler, M., Hedrich, H. J., Haagmans, B. L., Egberink, H. F., Horzinok, M. C. *et al.* (1997). Persistence and evolution of feline coronavirus in a closed cat-breeding colony. *Virology* **234**, 349–63.
19. Rottier, P. J. M. (1999). The molecular dynamics of feline coronaviruses. *Veterinary Microbiology* **69**, 117–25.
20. Wesley, R. D. (1999). The S gene of canine coronavirus, strain UCD-1, is more closely related to the S gene of transmissible gastroenteritis virus than to that of feline infectious peritonitis virus. *Virus Research* **61**, 145–52.