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PCR assay for the detection and the identification of atypical canine coronavirus in dogs

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Abstract

Comparative sequence analysis of the PCR products of the M gene and fragments of the *pol1a* and *pol1b* genes of canine coronavirus (CCoV) have demonstrated that two separate clusters of CCoV are present in dogs. This note describes a PCR assay to identify atypical CCoV strains with nucleotide substitutions in the M gene. A total of 177 faecal samples from dogs CCoV positive previously with the PCR assay were analysed. Sixty-two of the 177 samples were amplified with the PCR described in the present study and were thus considered atypical CCoVs. The specificity of the PCR typing assay was confirmed by sequence analysis of the PCR products.

Keywords: Dog; Coronavirus; PCR; Genotypes

1. Introduction

The *Coronaviridae*, a family of viruses belonging to the order of the *Nidovirales* (de Vries et al., 1997; Siddell, 1995; Enjuanes et al., 2000), can be grouped into three clusters on the basis of genetic comparison. Canine coronavirus (CCoV) is included in group I together with transmissible gastroenteritis virus (TGEV) of swine, porcine epidemic diarrhoea virus (PEDV), feline coronaviruses (FCoVs) and human coronavirus 229E (HCoV 229E). CCoV is an enveloped (+) RNA virus that is 27–32 kb in length (Siddell, 1995) and causes moderate or severe enteritis in pups. The 5′ two-thirds of the genome is occupied by open reading frames (ORFs) 1a and 1b, the expression of which yields large polyproteins. Downstream to ORF1b, there are smaller ORFs which encode for the structural proteins S, E, M, for the nucleocapsid (N) protein and for a number of presumptive non-structural proteins (Luytjes, 1995). Virus isolation onto cell cultures and electron micro-

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scopic examinations appear to be useful tools, for the diagnosis of CCoV infection. Therefore, CCoV is difficult to isolate in vitro and the common presence of coronavirus-like particles in faeces requires confirmation by other diagnostic methods.

Recently, a nested-PCR assay on a fragment of the gene encoding for the M protein was developed for the diagnosis of CCoV infection. The test proved to have a high sensitivity and specificity (Pratelli et al., 1999). The results obtained in another investigation also suggested that the role of CCoV as enteric pathogen has been underestimated and that the virus is largely widespread in the dog population (Pratelli et al., 2000).

A subsequent study revealed nucleotide variability in the sequence of the gene encoding for the transmembrane protein M of CCoV (Pratelli et al., 2001a). By comparative sequence analysis of the M gene and fragments of the *pol1a* and *pol1b* genes, it has been observed that CCoVs segregated in two separate clusters. The first cluster was intermingled with reference strains of CCoV genotype and therefore could be assigned to this genotype. The second cluster segregated separately from CCoV and FCoV genotypes and therefore these isolates may represent genetic outliers (Pratelli et al., submitted for publication). Recently, severe enteritis has been described in two different pups which had been caused by CCoV, as confirmed by PCR. The viruses, unfortunately, were not isolated in cell culture. The sequence analysis of the PCR products of the M and S genes carried out on the faecal samples from the two pups confirmed that the FCoV-like CCoV had caused the disease (personal observations).

It has been demonstrated that CCoV is particularly difficult to isolate in cell cultures (Tennant et al., 1994; Pratelli et al., 2000); this both hampers the acquisition of fundamental information on the pathogenetic role of CCoV in dogs and means that FCoV-like CCoV can be identified only after sequence analysis.

On the basis of these preliminary results, a PCR assay was developed to detect and identify the FCoV-like CCoV strains from faecal samples of infected dogs.

2. Materials and methods

2.1. Clinical specimens

A total of 177 faecal samples from dogs, about 2–10 months old, with enteritis and positive for CCoV by standard PCR (Pratelli et al., 1999) were used to develop a PCR assay for detecting FCoV-like CCoV. The samples were collected over a period of 2 years from either housed dogs (114 specimens) or dogs living in kennels (63 specimens) where periodical outbreaks of CCoV enteritis had been described. The pups showed moderate to severe enteritis and their faecal samples were negative for canine parvovirus (CPV2) by the haemoagglutination test. The samples were stored immediately at −20 °C until tested.

Three additional coronavirus strains were also examined: feline infectious peritonitis virus (FIPV), isolated from a cat with clinical signs of disease (Buonavoglia et al., 1995), FCoV type II (79-1683 strain) and TGEV (Purdue strain).

2.2. PCR assay

Viral RNA was extracted from clinical specimens using the RNeasy kit (Qiagen GmbH, Germany). The target sequence is a fragment of the gene encoding for the membrane protein M of CCoV, as reported previously (Pratelli et al., 1999). The primer pair CCoV1/CCoV2 amplified a 409 bp fragment. For the n-PCR, the first amplicon was subjected to a second round of amplification using the CCoV2 and CCoV3 primers and the same PCR cycling procedure. In a previous study, similar nucleotide substitutions in the binding site of the internal primer CCoV3 used for the n-PCR were demonstrated in the sequence analysis of the PCR products from five faecal samples of pups with diarrhoea. These variations were assumed to affect partially the efficiency of the n-PCR amplification, with the production of an undetectable amount of c-DNA. Moreover, these nucleotide substitutions occurred consistently in all five samples examined (Pratelli et al., 2001a) and the sequence and phylogenetic analyses performed on the entire gene identified the viruses

as FCoV-like CCoV. Based on these preliminary results an internal primer flanking the variable region (CCoV1a) was chosen on the basis of the mismatch between the typical CCoV and FCoV-like CCoV strains (Fig. 1). PCR was then performed with the primer pair CCoV1a/CCoV2,

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Insavc    : CAACTGTTACATTTATACTTTGGATTATGTATTTTGTTAGATCCATTCAGTTATACAGAAGGACTAAGTCTTGGTGGTCTT :  81
S-378     : ...T.............................A.................................................. :  81
C-27/3*   : ..GT.........GC.....................C.....T......C.............................. :  81
A-32/99*  : ..GT.A........GC.....................A.....T......C....T....................... :  81
79-1683   : ..GT...A......GC.........G...........G.....T......C....T........C..A..A.......... :  81
79-1146   : ..GT...A..G...GC.........G...........G.....TG.....C....T.....A..C..A..A.......... :  81
TN406     : ..TT...A..G...GC.........G...........G.....T......C....T........C..A..A.......... :  81
UCD1      : ..GTCA.A..G...GC...C.....G...........G............C....T........C..A..A.......... :  81
Purdue    : ...T..........G....C.................A..............G............................. :  81

Insavc    : TCAACCCTGAAACTAGCGCAATTCTTTGCGTTAGTGCGTTAGGAAGAAGCTATGTGCTTCCTCTTGAAGGTGTGCCAACTG : 162
S-378     : .T.............A.....................A..............C.......................... : 162
C-27/3*   : ....T..........AT...........T........AC.T.....................ACA.......... : 162
A-32/99*  : ....T.....G....AT........A............C.T.....................ACA.......... : 162
79-1683   : .T.............AT...........T....AC.....G..T..........TT.G..C.....T..CACA..C..G. : 162
79-1146   : .T..T.....G....AT...........T....A...A..G..T.....T..........CT.A..T...ACT..T..A. : 162
TN406     : .T..T.....G..C.A............T....A...A..G..T.....T.....T..A..CT.A..T...ACA.....G. : 162
UCD1      : .T.............AT.....C.....T....A...AC.G..T.....G.....T.....C.....T..CACT..C..A. : 162
Purdue    : ...............AA.....................A..............C.......................... : 162

Insavc    : GTGTCACTCTAACATTGCTTTCAGGGAATTTGTGTGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACATCGACAATTTAC : 243
S-378     : ...................C.............A.................................................. : 243
C-27/3*   : ....T.....T..T..A..A.....A...C.T.A.........A..T.....G..T.....A...........GC...... : 243
A-32/99*  : ....T.....T..T.....A.....A...C.T.A.........A..T.....G..T.....A...........GC...... : 243
79-1683   : ....T.....C..GC.A........A...C.A.A.........T..T.....G..T.....C.C.C...T..AC...... : 243
79-1146   : ....T..C..T..TC.A........A...C.A.A.........T........G..T......T.A.C......GC....G. : 243
TN406     : ..........C..T........G..A..CC.A.A.........T..T.....G..T......C.T.C......GC....G. : 243
UCD1      : ..........T..T...........A...C.A.A.........T..T..G..G..T......C.T.C......GC....G. : 243
Purdue    : ............T....................AC.................................................. : 243

Insavc    : CAAAATATGTAATGGTTGCATTACCTGTCAGAACCATAGTCTACACACTTGTTGGCAAGAAATTGAAAGCAAGTAGTGCAA : 324
S-378     : .......C.................AG...G.....T.....................................G. : 324
C-27/3*   : .......C.................AGT.....T..T.................A...C..................... : 324
A-32/99*  : .......C........C......T..AGT.....T..T.................A...C..................... : 324
79-1683   : .T.....C..C...A....TACG...AAT........C..T......T.A.....A..AC...........C..C...C. : 324
79-1146   : .T.....C..C...A....TAC....AGT........C..T..T...T.A.....A..AC....A.......C..C...C. : 324
TN406     : .T.....C..C...A....TAC....AGT........C..T......T.G.....A..AC....A.......C..C...C. : 324
UCD1      : .T........C...A....TAC....AGT........C..T......T.A.....G..AC....A.......C..C...C. : 324
Purdue    : .......C.................AG...G..T..T.....................G.............G. : 324

Insavc    : CAGGATGGGCTTACTATGTAAAGTCTAAAGCTGGTGATTAC : 365
S-378     : ......................A.................. : 365
C-27/3*   : ......................A.................. : 365
A-32/99*  : ......................A.................. : 365
79-1683   : .G....................A........C......... : 365
79-1146   : ................C.....A.................. : 365
TN406     : .G....................A.................. : 365
UCD1      : ..................C..A.................. : 365
Purdue    : .T....................A.................. : 365
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Fig. 1. Alignment of a fragment of the M protein nucleotide sequences of some coronavirus strains: CCoV Insavc (accession number: D13096), FCoV 79-1146 (accession number: AF326575), FCoV 79-1683 (accession number: Y13921), FCoV TN406 (accession number: X90570), FCoV UCD1 (accession number: X90575). Asterisks indicate FCoV-like CCoVs (Pratelli et al., 2001a). The binding site of primer CCoV1a is boxed.

where the sense primer CCoV1a was able to detect these atypical CCoVs.

While the primer pair CCoV1/CCoV2 amplifies a 409 bp sequence of the M gene, a 239 bp band is obtained with the primer pair CCoV1a/CCoV2 if the target sequence of FCoV-like CCoV is present in the sample. The sequence of the primers and their positions in the M gene segment are displayed in Table 1.

The reverse transcription was carried out in a total reaction volume of 20 μl containing PCR buffer 1 × (KCl 50 MM, Tris–HCl 10 mM, pH 8.3), $MgCl_2$ 5 mM, 1 mM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), RNase 1 U, MuLV reverse transcriptase 2.5 U, random hexamers 2.5 U. Synthesis of c-DNA was carried out at 42 °C for 30 min, followed by a denaturation step at 99 °C for 5 min. The mixture was brought up to a total volume of 100 μl, containing PCR buffer 1 × , $MgCl_2$ 2 mM, *Amplitaq Gold DNA polymerase* 2.5 U and 50 pmol of each primers CCoV1a (sense primer) and CCoV2 (antisense primer). Amplification was carried out under the following PCR conditions: 35 cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The final products were detected by gel electrophoresis, ethidium bromide staining and UV light transillumination.

3. Results

One hundred and seventy-seven faecal samples of dogs with diarrhoea were examined. Of these, 115 samples that had showed the conventional fragment (409 bp) of the typical CCoV strain, were not amplified with the primer pair CCoV1a/CCoV2. Instead, FCoV-like CCoVs were found in 62 faecal samples, generating a 239 bp fragment. All 62 specimens had been collected from dogs in two separate kennels, where periodical outbreaks of CCoV enteritis had been observed. The specificity of the PCR typing assay was confirmed by sequence analysis of the PCR products.

As expected, the FIPV, FCoV type II 79-1683 and TGEV Purdue strains were amplified by the primer pair CCoV1/CCoV2, but they were not amplified by the PCR typing test with the primer pair CCoV1a/CCoV2.

Table 1
Primers used for PCR amplification

Primer	Sequence 5′→3′	Sense	Position
CCoV1[a]	TCCAGATATGTAATGTTCGG	+	6729–6748
CCoV2[a]	TCTGTTGAGTAATCACCAGCT	–	7138–7118
CCoV1a	GTGCTTCCTCTTGAAGGTACA	+	6900–6920

[a] Pratelli et al. (1999).

4. Discussion

CCoV is an enteric pathogen of dogs responsible, in young pups, for mild or severe symptoms characterized by diarrhoea, vomiting, dehydration, loss of appetite and occasionally death. CCoV shedding in faeces occurs over a range of 6–14 days post-infection (Keenan et al., 1976; Tennant et al., 1991), although virus faecal shedding in infected pups has been detected by n-PCR for periods of up to 37 days (Pratelli et al., 2001b). The detection limits of virus isolation have been overcome by the development of PCR and n-PCR for the diagnosis of CCoV (Bandai et al., 1999; Pratelli et al., 1999; Naylor et al., 2001). Furthermore, sequence and phylogenetic analyses of the M gene clearly confirm that two different clusters of CCoV circulate in the dog population (Pratelli et al., submitted for publication). To date, the sequence analysis is the only available method to identify the two genotypes of CCoV. To overcome this diagnostic limit, a PCR assay has been developed for the rapid identification of FCoV-like CCoV from faecal samples of naturally infected pups. This test may be instrumental in furthering epidemiological studies on the occurrence and distribution of CCoV strains in dog population.

Feline coronavirus strains (FCoV 79-1683 and FIPV) and the TGEV Purdue strain were not amplified by this PCR typing assay, because, as observed in the sequence and comparative analysis of the amplicons from typical CCoV, FCoV-like CCoV, FCoV and TGEV strains, several nucleotide substitutions are present in the binding site of

the sense primer CCoV1a, thus affecting the efficiency of the PCR amplification (Fig. 1). The PCR assay described in the present study for the identification of FCoV-like CCoV strains also shows a very high specificity.

In conclusion, a PCR typing test has been described for the detection and identification of FCoV-like CCoVs confirming that this new genotype may cause occasional enteritis in pups and is particularly common in kennel populations thereby causing epizootic infections.

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