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Diagnosis of canine coronavirus infection using nested-PCR

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Abstract

The results of polymerase chain reaction (PCR) and nested polymerase chain reaction (n-PCR) assays for the diagnosis of canine coronavirus (CCV) infection, and the comparison with other diagnostic techniques, such as electron microscopy (EM) and virus isolation using A-72 cell line are reported. The study was carried out on 71 faecal samples of pups with enteritis. Of 71 samples examined 14 were positive in PCR, whereas 30 samples resulted positive in the n-PCR assay. CCV was detected by EM examination in only four out of 45 samples, and by virus isolation in three out of 30 samples n-PCR positive.

Keywords: Canine coronavirus; Diagnosis; Nested-polymerase chain reaction

1. Introduction

Canine coronavirus (CCV) is a single-stranded RNA virus belonging to the Coronaviridae family and is responsible for general mild gastro-enteritis in pups (Binn et al., 1974). Infected dogs shed CCV in faeces for 6–9 days (Keenan et al., 1976) but shedding can be prolonged in some pups. The virus content of faeces is very high at the time of clinical signs (Appel, 1987).

Electron microscopic (EM) examination of faecal suspensions or isolation in tissue cultures are the most commonly used techniques for diagnosis of the infection in dogs. However, both methods may be carried out only in specialised laboratories, and they are difficult and time-consuming.

Recently, the nested-polymerase chain reaction (n-PCR) assay was developed for the diagnosis of CCV infection, that was highly sensitive and specific (Pratelli et al., 1999). The results of n-PCR are reported and compared with other techniques for the diagnosis of CCV infection, such as EM and virus isolation.

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2. Materials and methods

2.1. Clinical specimens

Seventy-one faecal samples from pups with diarrhoea, 2–6 months old, found previously as negative by haemoagglutination test (EA) for canine parvovirus type 2 (CPV-2) infection, were examined. Clinical specimens were collected from dogs in the south and the north of Italy and from dogs imported from Hungary. The samples were stored immediately at −20°C until tested.

Several faecal samples from healthy pups without antibodies to CCV and living in kennels where no signs of clinical gastro-enteritis had been reported, were tested previously to evaluate the specificity of the tests employed in the present study.

2.2. Nested-PCR (n-PCR)

n-PCR was applied as reported previously (Pratelli et al., 1999). Briefly, faecal samples were diluted 1:100 in phosphate-buffered saline (PBS) and homogenised by vigorous vortexing. Insoluble components were removed by centrifugation for 5 min at $8000 \times g$, and genomic RNA was extracted using the RNeasy Total RNA kit (Qiagen GmbH-Germany) from 1 ml of the supernatant fraction of each sample.

The target sequence for amplification is a segment of the gene encoding for transmembrane protein M of CCV. The above sequence of 409 bp straddles nucleotides 337 and 746, as described by Herrewegh et al. (1998).

The following primers were applied:

CCV1: 5′-TCC AGA TAT GTA ATG TTC GG-3′ sense primer (337–356 nucleotides);

CCV2: 5′-TCT GTT GAG TAA TCA CCA GCT-3′ antisense primer (726–746 nucleotides);

CCV3: 5′-GGT GTC ACT CTA ACA TTG CTT-3′ internal primer (535–556 nucleotides).

2.3. Specificity of the amplicon

The specificity of the PCR amplified product was evaluated by sequencing with Abi Prism (Model 377-Version 3.0-ABI 100-Version 3.0).

2.4. Virus isolation

Attempts were made to isolate the virus either from faecal samples n-PCR positive (n.30) and from n-PCR negative samples (n.41). Specimens were homogenised (10% W/V) in Dulbecco-Minimal Essential Medium (D-MEM) and centrifuged at $4000 \times g$ for 20 min at +4°C. The supernatant of each sample was treated with antibiotics (5000 UI/ml penicillin, 2500 μg/ml streptomycin, 10 μg/ml amphotericin) for 30 min at 37°C, inoculated onto partially confluent monolayer of A-72 as described previously (Tennant et al., 1991) and then incubated at 37°C in a 5% CO_2 incubator. Each sample was considered negative for CCV if, after three passages, no cytopathic effect (cpe) was observed.

2.5. Electron microscopy

Negative staining EM observation was carried out on faecal samples or gut contents of 45 out of 71 pups. Samples were suspended in distilled water (10% v/v) and shaken immediately using a vortex. They were then frozen and thawed twice, the supernatant was harvested and centrifuged at $4000 \times g$ for 20 min. and at $9300 \times g$ for 10 min for clarification. The second supernatant (85 μl) was then ultracentrifuged in Airfuge Beckman for 15 min at 21 psi ($82000 \times g$). The Airfuge was fitted with an A 100 rotor holding six 175 μl test tubes in which specific adapters were placed for 3 mm grids which allowed direct pelleting of viral particles on carbon-coated Formvar copper grids.

Immune electron microscopy (IEM) was performed using a dog serum monospecific to CCV. The optimal working dilution of serum (1:80) was established testing a CCV positive sample with two-fold dilutions. An equal amount (50 μl) of the supernatant from the second centrifugation and of the optimal dilution was incubated at 37°C for 1 h before being ultracentrifuged.

Negative staining was carried out using 2% sodium phosphotungstate (pH 6.8). Examination was made using a TEM Philips CM10 operating at 80 kV at 19 000–39 000 magnification.

3. Results

DNA sequence analysis of 409 bp PCR products showed the fragment of strains tested to have 94% nucleotide sequence identity to CCV Insavc (reference strain). Sequence and alignments are not shown.

As showed in Table 1, only 14/71 (19.71%) of the samples examined were positive in the PCR assay; in contrast, 30/71 (42.25%) of the samples were positive in the n-PCR assay. The n-PCR positive samples included the 14 PCR positive samples. CCV was isolated in cell culture from only three of the 30 n-PCR positive samples.

Negative staining ultramicroscopic examination of the 45 samples showed viral particles resembling coronavirus morphologically in four cases. The virions were pleomorphic, 90–120 nm in diameter and had the typical spikes. Using IEM some groups of aggregated particles covered by a fuzzy halo of antibodies were seen. Two of those samples resulted positive in isolation assay, while the third and fourth were negative by virus isolation. Three out of the four EM positive samples were positive by PCR and n-PCR.

4. Discussion

Recently, CCV has emerged as an important pathogen responsible for variable, sometimes severe, enteritis in pups. Of the several methods used for the detection of CCV, electron microscopy, using negatively stained faecal samples, appears to be an essential diagnostic tool (Hyatt, 1989). EM has been reported to be more sensitive and useful than virus isolation for detecting both coronaviruses and rotaviruses (Naeem and Goyal, 1988). However, the common presence of coronavirus-like particles in faeces presents difficulties in the diagnosis of CCV by EM and requires confirmation by other diagnostic methods (Athanssious et al., 1994). n-PCR, on the other hand, allows the diagnosis of CCV infection more rapidly than the traditional tests, such as virus isolation, and would be valuable for the diagnosis of CCV in faecal samples where CCV is inactivated, or when the number of virions is less than 10^6 per g of faeces and cannot be detected by EM examination.

Table 1
Results of CCV detection in pup faecal samples

No. of samples	PCR[a]	n-PCR[b]	VI[c]	EM[d]
71	14/71[e]	30/71[e]	3/71[e]	4/45[e]

[a] Polymerase chain reaction.
[b] n-PCR.
[c] Virus isolation.
[d] Electron microscopy.
[e] Number of positive samples/number of samples examined.

The present study revealed a substantial difference between the results obtained with n-PCR and virus isolation or EM examination. Mochizuchi (1998) obtained similar results, where CCV was detected by PCR in 16/100 fresh faecal samples, but the virus was recovered from only two samples.

The detection limits of viral isolation is probably due to the low number of virus particles in the faeces at the time of sampling, or may be attributable to the pH, or to the presence of ions and colloids in faeces which might inactivate the virus (Tennant et al., 1994).

The results of the present study, if confirmed by further investigations, suggest that the role of CCV as an enteric pathogen may be underestimated since faecal samples from pups with gastroenteritis might be negative for both viral isolation and EM examinations, yet positive by n-PCR. Our n-PCR results revealed that CCV is widespread in the dog population and might be responsible for a higher frequency of enteritis in pups than reported. Our data concerning CCV genome prevalence in dog faeces confirms serological studies where antibodies were reported in approximately 60–80% of dogs in kennel populations (Appel, 1987).

n-PCR assay for the diagnosis of CCV revealed an apparent high sensitivity when compared to the conventional methods (virus isolation on cell cultures and EM), and allows results to be obtained rapidly. Our studies, which will be confirmed on faecal samples from experimental infected pups, suggest that the test may be useful for CCV diagnosis.

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