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Short Communication

Digital PCR Platform as a Tool to Determine the Canine Coronavirus (CCoV) Genome in Clinical Samples

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Abstract

Canine coronavirus (CCoV) is an important pathogen that affect dogs. Here we assessed a digital real-time based PCR (dPCR) for the detection of CCoV from infected AF-72 cells and directly from faeces from 100 symptomatic dogs. The results obtained from dPCR were compared to real-time TaqMan® based PCR assay (qPCR) and positive samples were submitted to phylogenetic analysis. Thus, dPCR had an equal sensitivity, 101 copies/μl of partial M CCoV gene, when compared to qPCR results with a good agreement in all analysis. These results indicated that the dPCR could be an alternative technique for the diagnosis of CCoV from clinical samples with advantage of simplicity and sensitivity.

Introduction

Canine coronavirus (CCoV) and coronaviruses of cats and pigs are closely and compose a unique viral species [1-3]. To date, two CCoV genotypes are known, designated types I and II, and canine/porcine recombinant viruses have also been identified [3]. Moreover, CCoV-II has been subdivided into CCoV-IIa (which derives from recombination with feline coronavirus; FCoV-II) and CCoV-IIb (which derives from recombination with porcine transmissible gastroenteritis virus; TGEV) with no association with clinical disease in dog [4,5]. However, a highly virulent strain (pantropic CCoV-IIa) was isolated during an outbreak of fatal, systemic disease in puppies in Italy [6]. Upon the emergence of pantropic CCoV-IIa strains, monitoring of these novel coronaviruses in dog populations has become more important [7-10].

The diagnostic of CCoV, due to difficulty of virus isolation and electron microscopy of viral particles from clinical samples, is based on virus molecular detection [11-12]. First description of quantitative real-time TaqMan® RT-PCR (reverse transcriptase-polymerase chain reaction) to detect and quantify RNA CCoV revealed high sensitivity and simplicity [13]. Driven by its potential benefits over currently available methods, and the recent development of commercial platforms, digital polymerase chain reaction (dPCR) has received increasing attention in virology research in order to quantify nucleic acids [11]. Moreover, dPCR, a newer technology than qPCR, provides absolute quantification without need for standard curves as well as high sensitivity, reproducibility and semi-automation [12].

Thus, this study aimed to investigate CCoV in domestic dogs, by the use of digital RT-PCR approach in clinical samples. For this purpose, virus isolation, conventional real time RT-PCR detection and sequencing analysis were performed, and the results compared.

Materials and Methods

Samples and Virus Isolation

One hundred faecal samples (n=100) from dogs presenting diarrhoea (symptomatic) were collected for this study. All samples were from client-owned puppies, less than 1 year of age and with unknown vaccination status, São Paulo State, Brazil. Samples were obtained inside each owner's house after mechanical remove

of faeces using gloves and stored under 4°C until sent to the Laboratory of Animal Virology at University of São Paulo. The samples were stored at -86°C prior to virus isolation and after for CCoV genome detection. All applicable institutional guidelines for the care and use of animals were followed (CEEA 2015/09754). In the laboratory, approximately 2 g of faeces was homogenized in 1 volume of sterile phosphate-buffered solution (PBS), clarified by centrifugation at 2,500 x g for 10 min. The supernatant was filtered through a 0.75 µm filter (Millipore™) and then treated with antimycotic/antibiotic 1 X solution (concentrated at 100X; Sigma-Aldrich®, St. Louis, MO, USA). AF-72 cells (ATCC, CRL 1542) were cultured in MEM (Sigma-Aldrich®) with 10% foetal calf serum (Sigma-Aldrich®), 2 mM L-glutamine (Sigma-Aldrich®), and non-essential amino acids (100 x, Invitrogen®, Life Technologies, Carlsbad, CA, USA). Cultures were incubated at 37°C in 5% CO_2 with 95% humidity. After AF-72 cells reached 80% confluence, 1 ml of faecal preparation was added to 4.7 x 10^5 cells/ml and submitted to three blind passages at 5-day intervals. Inoculated and control cells were monitored under phase-contrast using an Olympus IX-70 microscope for production of cytopathic effect (CPE) (Olympus®, Tokyo, Japan). Approximately 10 fields were analysed in each condition, and photographs were taken at 200 x magnification by using cell Sens™ software (Olympus®). CCoV VR-809™ (ATCC), strain 171 was used as control.
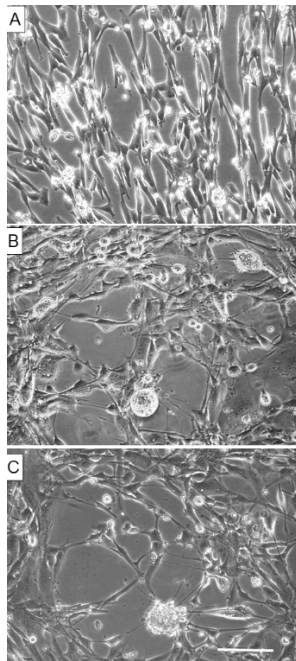
Molecular Analysis

Clinical samples, consisted of original faecal specimens and faeces suspension submitted to AF-72 cells infection, were submitted to total RNA extraction using the TRIzol® reagent protocol, according to manufacturer's instructions (Invitrogen®, Thermo Fisher, Carlsbad, CA, USA). A total of 2 ng of each RNA sample was reverse-transcribed using the High-Capacity RNA-to-cDNA™ Kit (Invitrogen®, Applied Biosystems™). The same primers and probe set were used in both dPCR and real time TaqMan® based quantitative PCR (qPCR) as described previously with some modifications [13]. The primers and probe were commercially prepared in a single tube assay by Applied Biosystems customers service: CCoV forward 5`-TTGATCGTTTTTATAACGGTTCTACAA-3`; CCoV reverse 5`- AATGGGCCATAATAGCCACATAAT- 3` and CCoV probe 5`- FAM-ACCTCAATTTAGCTGGTTCGTGTAT-GGCATT-MGB [13]. In order to normalize the qPCR a housekeeping gene 18Scf_forward 5`- TGCGAATGGCTCATTAAATC -3`; 18Scf_reverse 5`-CGTCGGCATGTATTAGCTCT- 3` and 18Scf_probe, 5`-FAM-TGGTTCCTTTGGTCGCTCGCT-MGB-3` was designed for this study. First, the qPCR reaction was conducted by OneStepPlus® PCR system (Applied Biosystems™) using 8µl of TaqMan® master mix, 300 nM of each primer and probe

and 8 ng of cDNA. The plates were sealed and loaded according to the following parameters: 96°C for 10 min, followed by 39 cycles of 60°C for 2 min and 98°C for 30 s and final extension at 60°C for 2 min. The cDNA and all reagents were tested by QuantStudio® 3D Digital PCR System (Thermo Fisher Scientific) after loaded onto chips with the same parameters described for qPCR. After cycling, the end-point fluorescence of the partitions on the chips was measured by transferring the chips to the measurement unit (version 1.1.3, algorithm version 0.13). For each reaction, three readings were used. The quality threshold was set to 0.5 in the colour by quality mode and an automatically calculated was used in the colour by calls mode to separate the positive and negative signals. To determine qPCR and dPCR performance, it was cloned a 409-bp cDNA fragment of the gene encoding transmembrane protein M from CCoV-IIa Insavc strain into SK-bluscript plasmid vector (psk; Agilent Technologies, Santa Clara, CA, USA), purified with Qiagen Miniprep Kit (Qiagen™, Hilden, Germany) and quantified. In order to obtain the 409-bp cDNA product PCR was performed with CCoVI-CCoVII (6729-7138) primer pair according to previous study [7]. A total of 10µl of a solution containing ten-fold scalar amounts of cloned plasmid (from 2 x 10^5 to 2 copies) were used to determine sensitivity of each assay. The specificity of both test was assessed with analysis of Canine Parvovirus (CPV) and Canine Distemper Virus (CDV), being the results negative for both viruses.
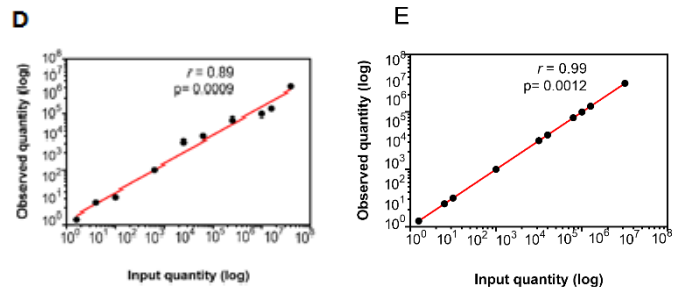
Results and Discussion

In order to characterize CCoV genotypes detected after virus isolation sequencing of S gene was performed as described previously [7,14]. CCoV amplicons were purified using the NucleoSpin Extract II kit and sequenced with an ABI PRISM 3100 Genetic Analyser (Applied Biosystems™) using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems™). Sequences were aligned using BioEdit Sequence Alignment Editor V.7.0.9.0 [15]. Phylogenetic analyses were conducted in MEGA4 and sequences generated for CCoV were submitted to GenBank and assigned. [16,17]. One-way ANOVA followed by Tukey´s Multiple Comparison Test were used. A value of $p < 0.05$ was considered statistically significant. All statistical analyses were performed using Prism software (GraphPad® v. 6.1, La Jolla, CA, USA).

All 86% samples from virus isolation produced cytopathic effect in AF-72 cells after three consecutive blind passages. The cytopathic effect was characterized by the appearance of floating, rounded cells detached from the monolayer (Figure 1A). Mock-infected AF-72 cells were used as negative controls (Figure 1B). AF-72 cells infected with CCoV VR-809™ (Figure 1C) were used as positive controls.

Figures 1(A-C): (1A) Cytopathic effects in AF-72-infected cells after three consecutive blind passages, as assessed by phase-contrast microscopy revealing floating rounded cells; (1B) CCoV VR-809™ cytopathic effect characterized by floating and rounded cells as described for the isolates. (1C) Uninfected AF-72 cells used as controls, bars-50μm.

To compare the analytical sensitivity, 10-fold dilutions of the standard RNA, ranging from 10^8 to 10^0 copies/μl, were tested by qPCR and dPCR, after RNA has been transcribed into cDNA. Each dilution was quantified three times separately. As shown, the detection limit of both assay was equal around 10^1 copies/μl (Figure 1D). The coefficient of linear regression (r^2) was lower (r=0.89) of qPCR in comparison to dPCR (r=0.99) (Figure 1D and E, respectively). The results obtained after AF-72 isolation 86 out of 100 fecal samples that produced cytopathic effect were considered positive for CCoV genome amplification in both molecular assay (Table 1). However, when fecal samples were directly searched for CCoV genome, dPCR was able to detect 4 samples as positive which were considered negative in qPCR analysis (Table 1). The coefficient of linear regression (r^2) was higher (r=0.86) when both methods were compared to detect CCoV from AF-72 isolated samples (Figure 2A). In addition, comparing qPCR and dPCR results obtained directly from fecal samples the linear regression was lower (r=0.72; Figure 2B). To construct the phylogenetic tree, the sequences of four viruses were included, representing the 87 sequenced from all analysed clinical samples. Sequences KR105601, KR105599, KR105604 and KR105600 clustered with sequences of S gene of 13 Brazilian viruses and genotypes from Italy and Greece [15,18]. The Brazilian samples tended to group into a single clade, suggesting a common ancestor, as described previously (Figure 2C).

Figures 1 (D-E): PCR platform observed in dilutions series of standard CCoV RNA and robust regression analysis reveal quantitative linearity of r=0.89 for qPCR (D) and 0.99 for dPCR (E), respectively.

	qPCR		dPCR	
	positive	negative	positive	negative
[a]AF-72 isolates (n=86)	86	0	86	0
Fecal samples (n=100)	68	32	72	28
[a]Virus suspension after cell blind passage that showed CPE (cytopathic effect). Numbers indicate the samples positive or negative obtained in both methods.				

Table 1: Cross-tabulation of the results of CCoV in AF-72 isolates and fecal samples analyzed by qPCR and dPCR.
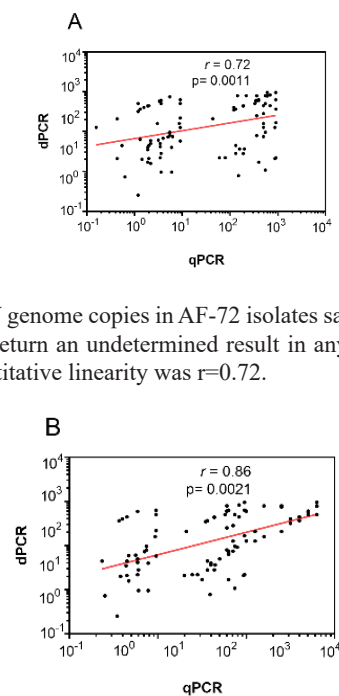

Figure 2A: CCoV genome copies in AF-72 isolates samples by both PCR methods did not return an undetermined result in any of the 86 positive samples and quantitative linearity was r=0.72.

Figure 2B: The same analysis when applied to detect CCoV genome directly from faeces showed r = 0.86 and undetermined results were found.
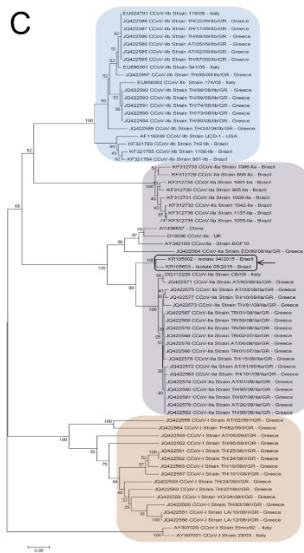
Figure 2C: Phylogenetic analysis of pantropic CCoV isolated in AF-72 cells based on partial sequencing of the S gene. Evolutionary distances were computed using the Tamura-Nei method and are expressed as the number of base substitutions per site. CCoVs detected in rural dogs in this study are denoted with arrows. Previously published CCoV sequences from different sources were included for comparative purposes.

A recent molecular characterization of CCoV in Brazil revealed that the sequences of genotypes I and II were similar to those circulating in Europe and China [15,18,19]. Regarding S gene sequences, CCoV IIa viruses isolated and sequenced in this study were similar to the CB/05 pantropic strain isolated in Italy from dogs presenting enteric disease [18]. Amplification of viral sequences was performed from nucleic acids extracted from AF-72 cells that showed CPE, in contrast with many studies that used DNA/RNA extracted directly from faeces [19].

Our study showed that isolation of CCoV is feasible when faeces are processed shortly after collection. This procedure allows for enriching viral preparations for several purposes, including biological studies of viral replication, drug susceptibility and virus-host interactions. CCoV isolation has been reported to be problematic due to the instability of viral particles under environmental conditions, so virus isolation succeeds only if samples contain high virus titres and are stored and transported under appropriately cold conditions [1,3]. The qPCR has been applied on experimental infected dogs and showed to have sensitivity in detect RNA from CCoV in different tissues described by previous studies [1-3,9,13]. The animal model to reproduce CCoV disease is infected dogs, fact that limited experimental studies [9,13]. However, in this study, it was not possible to trace a comparison between qPCR and dPCR in experimentally CCoV infected dogs due to animal care rules stablished in our institution. In this respect, only faeces obtained with non-invasive procedure is not allowed. Several studies have demonstrated the efficient dPCR platform searching different viruses [20-25]. Moreover, rapid, accurate and affordable molecular technology can be predictable with particular emphasis on emerging techniques (next generation sequencing, digital PCR, point of care testing and syndromic diagnosis) to simplify viral diagnosis in the next future [24,25]. Finally, the present findings show that dPCR enables direct and accurate detection of CCoV genome with efficiency and quantitative linearity both from fecal samples and infected cell suspensions.

Acknowledgments

Declaration of Conflicting Interests

The author (s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This manuscript does not contain any individual personal data, and individual consent to publish is not applicable.

Author Contributions

Conceived and designed the experiments: Tereza C Cardoso. Performed the experiments: Flavia V Vieira, Dielson S Vieira, Helena L Ferreira. Analyzed the data: Flavia V Vieira and Katia D S Bresciani. Contributed reagents/materials /analysis tools: Roberto Gameiro.

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