Development of a quantitative real-time PCR for the detection of canine respiratory coronavirus

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ABSTRACT

Canine respiratory coronavirus (CRCoV) has been detected recently in dogs with canine infectious respiratory disease and is involved in the clinical disease complex. CRCoV is a group 2 coronavirus most closely related to bovine coronavirus and human coronavirus OC43. A real-time PCR assay was developed for the detection of CRCoV. The assay was validated against cell culture grown virus and shown to have a high level of sensitivity. A range of tissue samples were collected from dogs at a re-homing centre with a history of endemic respiratory disease. The samples were tested using a conventional nested PCR assay and CRCoV was quantitated by real-time PCR. CRCoV was detected most frequently in the nasal mucosa, nasal tonsil and trachea. It was also detected in the lung, and bronchial lymph node. Of the enteric tissues, only one mesenteric lymph node sample was positive. In addition two colon samples were positive for CRCoV by nested PCR only. In conclusion, CRCoV appears to infect the upper respiratory tract preferentially. The CRCoV real-time PCR assay has proved to be a highly specific and sensitive assay that can be applied for diagnostic purposes as well as to investigate further the tissue tropism of CRCoV.

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1. Introduction

Canine infectious respiratory disease (CIRD) is a disease complex which occurs frequently in densely housed dog populations such as in re-homing, training and boarding kennels. The disease is characterised by a dry hacking cough with a recovery period of 1–3 weeks, but can progress to a severe bronchopneumonia which may be fatal (Appel and Binn, 1987). Many microbiological agents have been associated with CIRD; including *Bordetella bronchiseptica*, canine parainfluenza virus; canine adenovirus-2; canine herpesvirus and *Mycoplasma* spp. which may act alone or in synergy to cause disease (Binn et al., 1967; Ditchfield et al., 1962; Karpas et al., 1968; Keil and Fenwick, 1998; Randolph et al., 1993). However, despite widespread vaccination against a number of these agents, CIRD has remained a persistent problem (Erles et al., 2004).

Recently, a novel coronavirus; canine respiratory coronavirus (CRCoV); was detected in respiratory samples collected from dogs in a UK re-homing centre with a high incidence of CIRD (Erles et al., 2003), and the role of CRCoV in CIRD is currently under investigation.

Coronaviruses are large enveloped viruses containing a single stranded positive-sense RNA genome of approximately 30 kb which encodes a polymerase complex and several structural (spike (S), envelope (E), membrane (M), nucleocapsid (N), and in group two coronaviruses only a haemagglutinin esterase (HE)) and non-structural proteins. Currently coronaviruses are divided into three groups based on genetic and antigenic traits (Gonzalez et al., 2003).

Phylogenetic analysis places CRCoV in group 2 of the *Coronaviridae* family related most closely to bovine coronavirus (BCoV) and human coronavirus OC43 (OC43) (Erles et al., 2003). Serological studies have shown CRCoV to be present in the UK, Ireland, Italy, USA and Japan (Decaro et al., 2007; Erles and Brownlie, 2005; Kaneshima et al., 2006; Priestnall et al., 2006, 2007).

Current detection methods for CRCoV include virus isolation and detection of viral RNA by reverse transcription polymerase chain reaction (RT-PCR) (Erles et al., 2007, 2003). Cell culture isolation of CRCoV is difficult and time consuming, and therefore lacks the sensitivity and speed required for high-throughput screening (Erles et al., 2003; Kaneshima et al., 2006). Molecular assays such as RT-PCR are faster and offer increased sensitivity and specificity over other detection methods (Tang, 2003; Wang et al., 1999), therefore providing the best opportunity for the detection of CRCoV. The current RT-PCR method for the detection of CRCoV (Erles et al., 2003), however does not enable quantitation of viral load.

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Studies which focus on the assessment of tissue tropism, viral load and shedding are vital for understanding the pathogenesis of CRCoV. In a clinical setting this has particular relevance for monitoring the course of disease, disease management and clinical outcome, therefore the development of a quantitative PCR (qPCR) is essential.

Real-time qPCR has many advantages over conventional nested PCR methods. In addition to increased speed and often sensitivity, real-time qPCR offers the ability to quantitate viral load in a closed tube system which requires no further manipulation, therefore also reducing the potential contamination problems encountered commonly with nested PCR assays (Bustin, 2000; Bustin and Mueller, 2005). One qPCR method available currently, which may be used for the quantitation of CRCoV, uses highly degenerate primers which enables the detection of coronaviruses from all three coronavirus groups (Escutenaire et al., 2007). As such this assay is unable to distinguish between CRCoV and the other coronavirus which infects dogs, canine coronavirus (CCoV); a group one virus, which causes enteric disease (Binn et al., 1974).

The present study describes the development of a real-time qPCR for CRCoV and the quantitation of CRCoV RNA loads in various post-mortem tissues collected from dogs during naturally occurring infections with the virus. These data are important for defining the natural history and tropism of CRCoV infection, and improving understanding of the basic pathology of CRCoV.

2. Materials and methods

2.1. Cells and viruses

A 75-cm^2 culture flask containing HRT-18 cells (ECACC), was washed with 2 ml of serum-free RPMI containing 1 μg/ml trypsin (Sigma, Gillingham, UK) and inoculated with 1×10^6 TCID$_{50}$ of CRCoV isolate 4182 (Erles et al., 2007). After 1 h at 37 °C the inoculum was removed and replaced with 10 ml of serum-free RPMI containing 1 μg/ml trypsin. After 5 days the virus laden cell culture supernatant was titrated as described previously (Erles et al., 2007).

2.2. Study population and sample collection

The carcases of 10 dogs from a re-homing kennel were used in this investigation. One dog was suffering from cough and nasal discharge at the time of euthanasia. The other nine dogs did not exhibit obvious signs of respiratory disease or any other clinical conditions. Necropsies were performed within 4 h of death. Samples from the following tissues were harvested for storage at −70 °C: nasal mucosa, nasal tonsil, palatine tonsil, mid-trachea, right apical lung lobe, left diaphragmatic lung lobe, bronchial lymph node, liver, spleen, colon and anterior mesenteric lymph node (within the root of the mesentery). Each tissue sample was taken using a new set of sterile instruments to avoid cross-contamination. Tracheal samples collected from an additional 38 dogs from the same re-homing centre, all with evidence of respiratory disease, were also collected in the same manner. All the dogs used in this study were euthanized solely on grounds of being unsuitable for re-homing due to aggressive behaviour.

2.3. RNA extraction from cell culture derived virus

RNA was extracted using the RNeasy Mini Kit (Qiagen, Crawley, UK) from 200 μl of CRCoV infected cell culture supernatant samples as recommended by the manufacturer.

2.4. RNA extraction from clinical samples

RNA was extracted using TRiReagent (Sigma) from approximately 25–50 mg of homogenised canine tissue samples as recommended by the manufacturer. RNA was treated with RQ1 RNAse free DNAse (Promega, Southampton, UK) to remove genomic DNA according to the manufacturer's protocol.

2.5. cDNA synthesis and quantification

RNA was transcribed into cDNA using Random Hexameres (GE Healthcare, Little Chalfont, UK) and Improm II reverse transcriptase (Promega) according to the manufacturer's protocol. Total cDNA was quantified using the Quant-iT^{TM} PicoGreen® Assay Kit (Invitrogen, Paisley, UK) which selectively detects dsDNA (removed from these samples by DNase treatment (see Section 2.4)) and DNA–RNA hybrids (cDNA) (Seville et al., 1996). The assay was performed using a modification of a previously published protocol (Whelan et al., 2003). Briefly the λ DNA standard was diluted in 1× Tris–EDTA (TE) in a fivefold dilution series from 1 μg/ml to 1.6 ng/ml. cDNA samples were diluted 1:50 in 1× TE. 50 μl per well of each standard/cDNA sample was aliquoted in duplicate in a 96 well plate. The PicoGreen dsDNA quantitation reagent (Invitrogen) was diluted 1:200 in 1× TE and 50 μl was added to each well, therefore making a further 1:2 dilution of the standards and samples. The fluorescence was measured using the SPECTRAmax M2 Plate reader (Molecular devices) with an excitation at 480 nm and emission at 520 nm. The linear standard curve was constructed for the λ DNA standard concentration vs. fluorescence units. The concentration of the cDNA was calculated from the linear equation after adjusting for the dilution.

2.6. Conventional PCR for GapDH

Prior to analysis for CRCoV all samples were tested for the presence of the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GapDH) by conventional PCR as described previously (Grone et al., 1996). The PCR products were resolved on 1.5% agarose gels containing 0.5 μg/ml of ethidium bromide and visualised under UV illumination using Gel Doc 2000 (Bio-Rad, Hemel Hempstead, UK) for image capture.

2.7. Conventional CRCoV spike gene PCR

Clinical samples were analysed for CRCoV using the conventional nested spike gene PCR as described previously (Erles et al., 2003). The PCR products were resolved on 1.5% agarose gels containing 0.5 μg/ml of ethidium bromide and visualised under UV illumination using Gel Doc 2000 (Bio-Rad) for image capture.

2.8. Real-time qPCR primer design

Due to the limited availability of CRCoV sequence data the CRCoV nucleocapsid gene was selected as the target for the real-time PCR as it is highly conserved within each coronavirus group, thus ensuring the detection of variant CRCoV strains, whilst being sufficiently divergent to differentiate between the three coronavirus groups, and therefore CRCoV and CCoV (Gonzalez et al., 2003). The primers CRCoV NF3 (forward 5′-CCCTACTATTCTTGGTT-3′) and CRCoV NR4 (reverse 5′-CGTCTGTTGTGTCTGTACC-3′) were designed against the only available nucleotide sequence for the CRCoV nucleocapsid gene (GenBank accession no: DQ682406) which was aligned with sequences from other group 2 coronaviruses previously published in the GenBank database (GenBank accession no: U00735, AF220295, AY585228, AY585229, NC007732, DQ011855), a group 1 CCoV sequence (GenBank accession no: AY548235) and
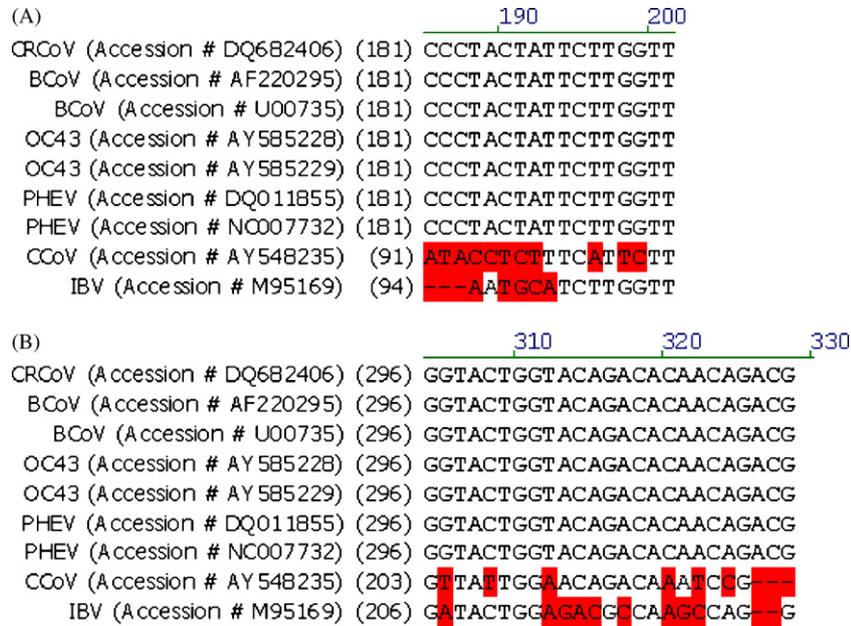
Fig. 1. Nucleotide alignment of qPCR primer binding sites. Nucleotide sequences of the (A) CRCoV NF3 and (B) CRCoV NR4 primer-binding sites. The alignment includes sequences from the group 2 coronaviruses CRCoV, BCoV (bovine coronavirus), OC43 (human coronavirus OC43), PHEV (porcine hemagglutinating encephalomyelitis virus), group 1 coronavirus CCoV (canine coronavirus) and group 3 coronavirus IBV (infectious bronchitis virus). The numbers in the brackets show the starting position of the primer-binding site within each sequence. The numbers along the top show the nucleotide positions within the alignment. Shaded areas indicate the location of the non-conserved nucleotides in CCoV and IBV.

a group 3 Infectious bronchitis virus (IBV) sequence (GenBank accession no: M95169). Sequences were aligned using the AlignX feature within Vector NTI™ Advanced 10 Software (Version 10.1.1 Invitrogen) (Fig. 1). The primers were assessed for their specificity using the database similarity search program Nucleotide Blast (www.ncbi.nlm.nih.gov/blast). The primer sequences showed no homology with any other region of the CRCoV genome. The primers were shown to be homologous with the nucleocapsid gene sequences of other group 2 coronaviruses, but not of groups 1 or 3 (Fig. 1).

2.9. Preparation of constructs as standards

For quantitation of CRCoV in clinical samples CRCoV nucleocapsid gene was amplified, cloned, and used to generate the standard curve. To prepare the standard control plasmid the 140 bp fragment of the CRCoV nucleocapsid gene was amplified from cell culture grown virus using the primers CRCoV NF3 and CRCoV NR4 and the following cycling conditions: 95 °C 1 min followed by 35 cycles of 95 °C 1 min, 45 °C for 40 s, 72 °C for 1 min, with a final extension of 72 °C for 10 min. PCR products were eluted from the agarose gel using the Qiaquick gel extraction kit (Qiagen) and cloned into the pGem®-T Easy vector (Promega). Plasmid DNA was purified using the GeneElute plasmid miniprep kit (Sigma). Clones were screened for the insert using the appropriate restriction enzymes, and positive clones were sequenced. DNA concentration of the plasmid preparation was determined and the copy number calculated using the following formula: (DNA concentration in $\mu g/\mu l \times 6.0233 \times 10^{23}$ copies/mol)/(DNA size (bp) $\times 660 \times 10^6$). A 10-fold dilution series of the plasmid DNA (1×10^8 to 1×10^1 copies/μl) was prepared and used to generate the standard curve.

2.10. Real-time PCR

Real-time PCR was performed using SYBR® Green Jumpstart Taq Ready Mix (Sigma) with final concentrations of 2.5 mM MgCl$_2$ and 0.4 μM of each primer. For each reaction 1 μl of cDNA template or standard was added to a total reaction volume of 25 μl. Samples were analysed in triplicate. In each run, the standard dilution series was included. The cycling conditions were 95 °C for 10 min, followed by 40 cycles of 94 °C for 10 s, 53 °C for 20 s, 72 °C for 30 s, and 79 °C for 10 s after which the plate read was taken. This was followed by melting curve analysis from 55 to 95 °C over 20 min and a re-annealing step of 72 °C for 5 min. All reactions were performed on a DNA Engine Opticon 2 (Bio-Rad).

Analysis of copy number, linear regression and melting curve analysis was performed for each experiment using the Opticon Monitor 2 Software version 3.1. PCR efficiency for each run was calculated using the following equation: % PCR efficiency = $[(10^{-1/-\text{Slope}}) - 1] \times 100$. Runs with PCR efficiency <90% and >110% were disregarded. Quantitative data was normalized by either sample volume or cDNA concentration.

3. Results

3.1. Optimisation of the real-time PCR

Optimisation of the real-time PCR reaction components and cycling conditions were undertaken using the DNA standards, cell culture grown CRCoV and clinical samples with known CRCoV status. The primers and magnesium chloride were titrated to determine the optimum concentrations. Different annealing and data acquisition temperatures were also evaluated (data not shown). The optimum conditions were defined as those which gave the maximum fluorescence and lowest Ct values without the presence of primer dimers or non-specific amplification.

3.2. Dynamic range, sensitivity and PCR efficiency

The dynamic range and efficiency of the assay was evaluated using several different templates, including a DNA standard diluted serially, DNA standard spiked into CRCoV negative cDNA, serially diluted cDNA from a CRCoV positive sample, and cell culture grown
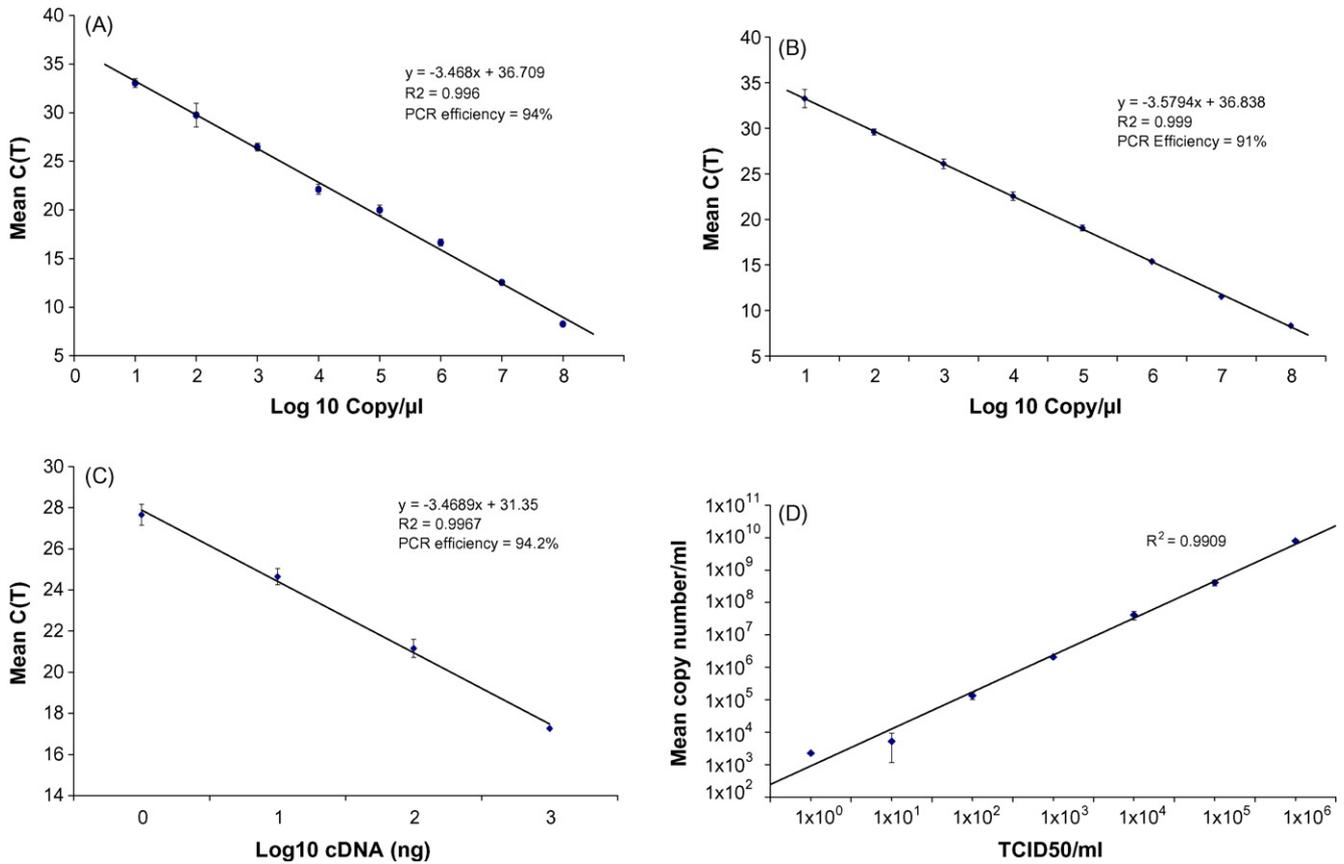
Fig. 2. Dynamic range, sensitivity and PCR efficiency. Mean Ct values vs. concentration of (A) serially diluted DNA standard; (B) serially diluted DNA standard spiked into CRCoV negative cDNA; (C) serially diluted cDNA from a CRCoV positive tracheal sample; and (D) TCID$_{50}$ of serially diluted cell culture grown virus vs. mean copy number/ml determined by the PCR in log scale. Error bars show ±1 S.D.

virus diluted serially. The results were analysed in terms of mean Ct value or copy number (Fig. 2).

Against the DNA standard and DNA standard spiked into cDNA the PCR was able to detect 10-fold differences in DNA concentration over a range of 1×10^1 to 1×10^8 copies/reaction (<1×10^1 and >1×10^8 not tested) with PCR efficiencies of 94% and 91%, respectively (Fig. 2A and B), indicating that the presence of cDNA is not detrimental to the efficiency or dynamic range of the assay. Analysis of the cDNA dilution series from a CRCoV positive tracheal sample showed that amplification from a cDNA target was as efficient (94.2%) as with plasmid DNA (Fig. 2C).

The sensitivity of the assay using a dilution series of cell culture grown virus with know viral titre (1×10^0 to 1×10^6 TCID$_{50}$) was also assessed. The qPCR was shown to be sensitive, with 1×10^0 TCID$_{50}$ yielding a mean copy number of 2.3×10^3 copies/ml. In addition there was a good correlation between viral titre and copy number observed, with an R^2-value of 0.99 (Fig. 2D).

3.3. Repeatability

Intra- and inter-assay variability was evaluated using the DNA standard. To assess intra-assay variation the DNA standard was tested at 1×10^2 and 1×10^6 copies/reaction in triplicate in a single assay. To assess inter-assay variation the DNA standard at 1×10^2 and 1×10^6 copies/reaction was tested in triplicate in six separate PCR assays. The mean, standard deviation (S.D.) and coefficient of variation (CV) of the Ct value and calculated copy numbers were determined. Overall intra-assay variability (Mean CV ± S.D.) was $0.97 \pm 0.13\%$ for Ct values and $13.88 \pm 7.24\%$ for calculated copy

numbers; whereas inter-assay variability was $2.56 \pm 1\%$ for Ct values and $24.39 \pm 2.04\%$ for calculated copy numbers (Table 1).

3.4. Detection of CRCoV in clinical samples: comparison of CRCoV conventional spike gene PCR and qPCR for the

To evaluate the use of the real-time qPCR for the detection of CRCoV in clinical samples, a range of tissues collected from 10 dogs at a re-homing centre with a history CIRD were analysed. Tracheal samples collected from an additional 38 dogs at from the same re-homing centre were also tested. The tissues were analysed for CRCoV using both the qPCR and the previously published conventional nested spike gene PCR (Erles et al., 2003). The results obtained

Table 1
Inter- and intra-assay variability for the CRCoV real-time PCR-based on Ct and calculated copy number values.

Variation		Coefficient of variation (%)	
	Copies/reaction	Cycle threshold (Ct)	Calculated copy number
Intra-assay	1×10^2	1.07	19.01
	1×10^6	0.88	8.76
	Overall CV	0.97	13.88
	Overall S.D.	0.13	7.24
Inter-assay	1×10^2	1.85	25.83
	1×10^6	3.28	22.95
	Overall CV	2.56	24.39
	Overall S.D.	1.01	2.04

Overall CV: overall coefficient of variation for the assay (CV = SD/mean). Overall S.D.: overall standard deviation for the assay.
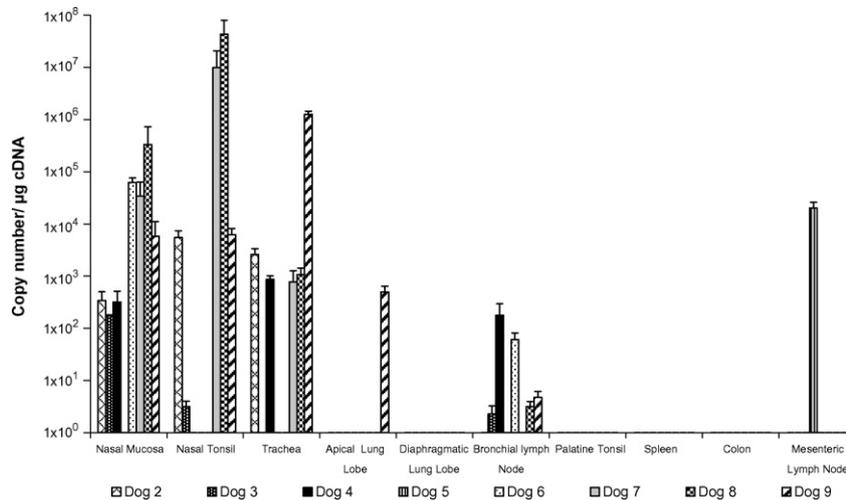
Fig. 3. Quantitative PCR analysis of CRCoV in canine tissues. Tissues from 10 dogs were analysed. The copy number of CRCoV was normalized to µg of cDNA. Error bars represent 1 S.D. All tissues from dogs 1 and 10 were negative for CRCoV.

by the two assays were compared. In addition, all samples yielded positive results for the house keeping gene GapDH.

CRCoV was detected in tissues from 8 out of the 10 dogs; all the tissues from dogs 1 and 10 were negative for CRCoV. CRCoV was detected most frequently in the nasal mucosa (7/10 dogs), nasal tonsil (5/10 dogs), trachea (5/10 dogs), and bronchial lymph node (5/10 dogs) by qPCR. It was also detected in the apical lung lobe of one dog (dog 9). Of the enteric tissues, CRCoV was detected in the mesenteric lymph node of one dog (dog 5) (Fig. 3). All samples positive for CRCoV by the real-time qPCR; with the exception of two bronchial lymph node samples (dogs 3 and 6); were confirmed as CRCoV positive using the conventional nested spike gene PCR. In addition, two colon samples (dogs 8 and 10) were positive for CRCoV by nested PCR but were negative by real-time qPCR.

Viral copy numbers ranged from 1.5×10^2 copies/µg of cDNA in the bronchial lymph node to 1.9×10^9 copies/µg of cDNA in the nasal tonsil (Fig. 3).

Of the 38 tracheal samples analysed, 30 were positive and 8 were negative for CRCoV by both real-time qPCR and conventional nested PCR.

Following the analysis of the 138 tissue samples the overall clinical sensitivity of both the real-time qPCR and the conventional nested PCR assays were comparable.

3.5. Specificity

Melting curve analysis of each sample showed that there was no evidence of non-specific amplification except in CRCoV negative spleen and nasal mucosa samples. These non-specific products had melting temperatures ranging from 76 to 79 °C (specific product Tm 82.5 °C), therefore, any false positive results could be disregarded through analysis of the melting curve (Fig. 4).

In addition, real-time qPCR was used to test samples known to be positive for CCoV. There was no amplification observed from these samples demonstrating the qPCR could be used specifically for the detection of CRCoV in canine samples (data not shown).

4. Discussion

The development and validation of a SYBR® Green based real-time qPCR assay are described for the detection and quantification of CRCoV. This assay has been validated for use in a range of canine
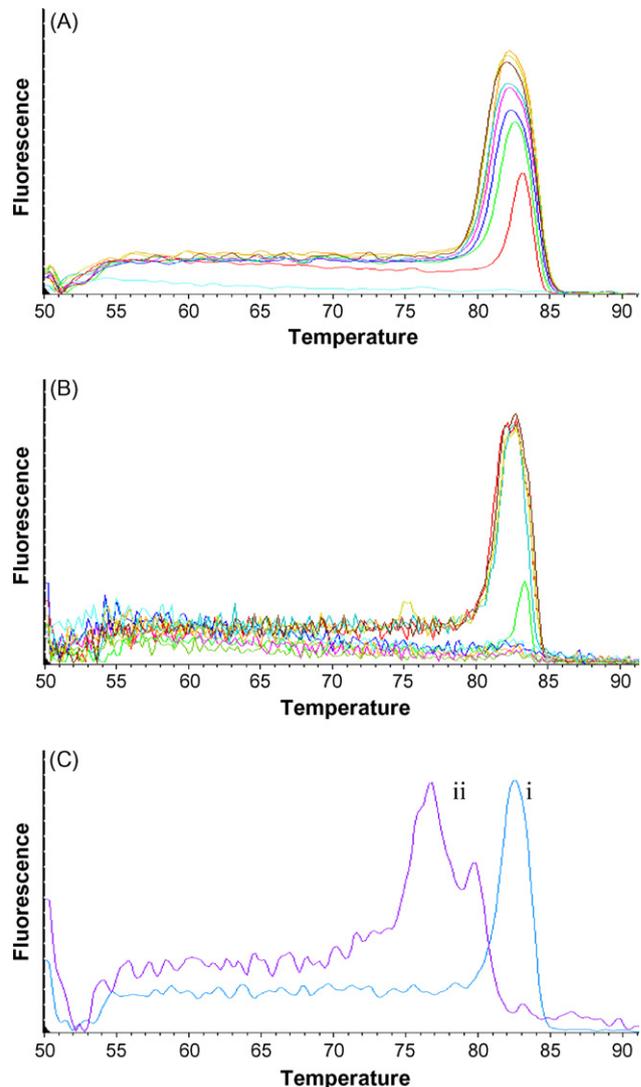
Fig. 4. Melting curve analysis. Melting curve analysis of (A) CRCoV qPCR standards, (B) five CRCoV positive and five CRCoV negative tissues, and (C) a (i) positive and (ii) negative nasal mucosal samples as an example of the non-specific amplification which occurs in CRCoV negative nasal mucosal and spleen samples.

tissues and respiratory samples and can be applied for diagnostic and research purposes.

The CRCoV N gene was quantified using a standard curve generated from a dilution series of plasmid DNA containing the N gene target sequence. This assay was shown to have a high degree of sensitivity and performs well over a wide dynamic range. The assay was able to detect 10-fold differences, over 8 logs, of plasmid DNA in concentrations down to less than 10 copies per reaction and less than 1 $TCID_{50}$ of cell culture grown virus. Furthermore, the presence of cDNA or the use of cDNA as the target did not adversely affect PCR efficiency or sensitivity when compared to plasmid DNA. The high sensitivity combined with its wide dynamic range makes it ideal for detecting high and low level infections.

The ability to detect low copy numbers is particularly important for assessing the true incidence and involvement of CRCoV in CIRD. Due to the complex and multifactorial nature of CIRD the ability to detect low copy numbers of virus, which may not be directly causing clinical illness is vital, as recent or low level infection may predispose animals to more severe disease.

In addition the data show that this assay was highly reproducible. When intra- and inter-assay variation of the Ct values (logarithmic units) were analysed, mean CV values of <1% and 2.6%, respectively, were obtained. The CV values obtained from calculated copy numbers (linear units) were <14% and 25%, respectively.

The assessment of tissue tropism and viral load are vital for a better understanding of the pathogenesis of CRCoV, and has important implications in disease management and outcome. With this in mind the ability of the real-time qPCR to detect and quantify CRCoV viral loads was assessed using a range of post-mortem tissues collected from dogs that were infected naturally with the virus.

Of the canine tissue samples analysed, CRCoV was found predominantly in tissues of the upper respiratory tract, with the largest quantities of virus being detected in the nasal tonsil. CRCoV was also detected in the bronchial lymph node, lung, and mesenteric lymph node. The clinical sensitivity of the real-time qPCR and conventional nested CRCoV PCR results were comparable. However, there were two bronchial lymph node samples which were positive for CRCoV by qPCR but negative by the conventional PCR, and two colon samples which were negative by qPCR but positive by the conventional PCR. This is most likely due to the low copy numbers present in the samples and indicates that both assays may give rise to false negative results. The presence of PCR inhibitors in these samples can be ruled out as GapDH RT-PCR results are positive for all the tissues analysed. Further tests using colon samples will be necessary to further validate the performance of the qPCR with this tissue. Assay specificity for the detection of CRCoV in tissues was high, with no evidence of non-specific amplification observed, except for CRCoV negative spleen and nasal mucosa. Nevertheless false positives generated in these tissues could be easily disregarded through analysis of the melting curve data.

This study confirms that CRCoV infections are not restricted to the respiratory tract. The virus was shown previously to be present in samples from the lung, spleen, mesenteric lymph nodes and intestines of one dog (Decaro et al., 2007). In addition CRCoV has been detected in a rectal swab from one dog (Yachi and Mochizuki, 2006). In an experimental study carried out using bovine coronavirus two dogs inoculated intra-nasally were found to be positive for BCoV in rectal swabs on days 1 and 5, respectively. Bovine coronavirus causes both respiratory and enteric disease in cattle and CRCoV may exhibit a similar tissue tropism. In the current study the samples were from dogs housed at a facility with a known high prevalence of CRCoV. Due to the collection of samples from dogs with naturally occurring infection, the choice of time points for sampling was limited and the duration of infection was unknown.

In order to determine the precise tissue tropism of CRCoV experimental studies will be required.

In conclusion, the SYBR green based real-time qPCR developed in this study was established for the detection and quantitation of CRCoV in infected animals, and applied to a range of canine tissues. It has proved to be reproducible, sensitive and specific over a wide dynamic range and will be useful for the analysis of canine tissues and clinical samples. This assay therefore will be an invaluable tool for further research and diagnostic purposes. In addition this report shows evidence of CRCoV in a range of canine tissues during infection. Tissue samples from the upper respiratory tract were found to be positive most frequently and also contained the highest copy numbers of CRCoV RNA; however further investigation is required to assess the true extent of CRCoV tropism.

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