Arch Virol (2007) 152: 41–58
DOI 10.1007/s00705-006-0840-x
Printed in The Netherlands

Archives of Virology

SYBR Green real-time reverse transcription-polymerase chain reaction assay for the generic detection of coronaviruses*

S. Escutenaire[1], **N. Mohamed**[2], **M. Isaksson**[1], **P. Thorén**[1], **B. Klingeborn**[1], **S. Belák**[1,3], **M. Berg**[3], and **J. Blomberg**[2]

[1]Department of Virology, National Veterinary Institute, Uppsala, Sweden
[2]Department of Medical Sciences, Section of Clinical Virology, Academic Hospital, Uppsala University, Uppsala, Sweden
[3]Department of Biomedical Sciences and Veterinary Public Health, Section of Parasitology and Virology, Swedish University of Agricultural Sciences, Uppsala, Sweden

Received May 4, 2006; accepted July 12, 2006
Published online August 28, 2006

Summary. Coronaviruses are etiologic agents of respiratory and enteric diseases in humans and in animals. In this study, a one-step real-time reverse transcription-polymerase chain reaction (RT-PCR) assay based on SYBR Green chemistry and degenerate primers was developed for the generic detection of coronaviruses. The primers, designed in the open reading frame 1b, enabled the detection of 32 animal coronaviruses including strains of canine coronavirus, feline coronavirus, transmissible gastroenteritis virus (TGEV), bovine coronavirus (BCoV), murine hepatitis virus (MHV) and infectious bronchitis virus (IBV). A specific amplification was also observed with the human coronaviruses (HCoV) HCoV-NL63, HCoV-OC43, HCoV-229E and severe acute respiratory syndrome coronavirus (SARS-CoV). The real-time RT-PCR detected down to 10 cRNA copies from TGEV, BCoV, SARS-CoV and IBV. In addition, the assay exhibited a high sensitivity and specificity on clinical samples from different animal species. The developed assay represents a potential tool for laboratory diagnostics and for detecting still uncharacterized coronaviruses.

Introduction

Coronaviruses are enveloped positive single-stranded RNA viruses, members of the order *Nidovirales* [8]. Their genome is 27–31 kb in length and is composed in its 5′-proximal two-thirds of two large open reading frames (ORFs), ORF1a and ORF1b, encoding the replicase complex [1]. Genes encoding the structural pro-

*The study was performed at the Department of Virology, SVA, Uppsala, Sweden.

teins, i.e. spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins, are downstream of the ORF1b. Coronaviruses are divided into three groups based on antigenic and genetic characteristics. Groups 1 and 2 infect a large range of mammalian species, whereas group 3 is restricted to birds [30]. Classification of the severe acute respiratory syndrome coronavirus (SARS-CoV) in group 2 or as the prototype of a new group 4 is subject to controversy and is complicated by the putative recombinant origin of its genome [12]. Coronaviruses are responsible for a broad spectrum of diseases, including respiratory and enteric pathologies, both in humans and in animals [30]. The importance of coronaviruses in public health has been revealed by the identification of SARS-CoV [6, 20, 26]. The virus emerged in China in 2002 and spread worldwide, causing a severe pneumonia in humans with 10% mortality out of more than 8000 cases [40]. More recently, human coronavirus NL63 (HCoV-NL63) and CoV-HKU1 have also been associated with severe lower respiratory tract diseases [9, 38, 41]. Although human infections with HCoV-OC43 or HCoV-229E are generally mild, both coronaviruses can be additional etiological agents of bronchiolitis and pneumonia [11, 37].

The emergence of pathogenic human coronaviruses has prompted research laboratories to set up fast and sensitive diagnostic assays. Real-time reverse transcription-polymerase chain reaction (RT-PCR) protocols based on different chemistries, including TaqMan probes or SYBR Green, are now available for the diagnostics of SARS-CoV, HCoV-NL63, CoV-HKU1, HCoV-OC43 or HCoV-229E infection [4, 5, 7, 9, 16, 17, 27, 39, 41]. The development of real-time RT-PCR tests to detect animal coronaviruses has been more limited, and protocols only apply to a restricted range of pathogens [2, 3, 13, 18, 32].

Although molecular techniques enable the detection of species or groups of coronaviruses, there are few reports of pancoronavirus RT-PCR, and none has been adapted to real-time format [20, 23, 33]. All methods are based on amplification of fragments from the replicase gene presenting highly conserved structure and function. This region is suitable to design primers allowing a broad-spectrum detection of genetically distant coronaviruses. Stephensen et al. [33] initially described a set of consensus primers targeting a 251-nucleotide (nt) fragment of the ORF1b. The primer sequences were subsequently modified for better reactivity, notably to the newly identified HCoV-NL63 [23]. Furthermore, Sampath et al. [31] recently reported a broad-range PCR targeting the same conserved region of the ORF1b followed by electrospray ionisation mass spectrometry and base composition analysis for viral identification.

The aim of the present study was the development of a real-time RT-PCR for the generic detection of coronaviruses, including still uncharacterized variants of human or animal origin. The method is based on SYBR Green chemistry and uses a new set of degenerate primers designed using conserved stretches of the ORF1b.

Materials and methods

Collection of coronavirus strains and clinical samples

A collection of coronaviruses was constituted to assess the efficacy of the real-time RT-PCR (Table 1). Thirty-six human or animal strains were subjected to the analysis. Due to

Table 1. Human and animal coronaviruses subjected to the SYBR Green real-time reverse transcription-polymerase chain reaction

Coronavirus species	Strain	Source	Threshold cycle (Ct)
Group 1			
HCoV-NL63	NL63	L. van der Hoek	25.2
HCoV-229E	229E	ATCC	14.3
CCV type I	Elmo/02	C. Buonavoglia	30.0
CCV type II	na	I. Kiss	16.5
CCV type II	144/01	C. Buonavoglia	16.2
FCoV type I	FIPV UCD-1	I. Kiss, P. Rottier	40.7
FCoV type I	FIPV UCD-2	I. Kiss	35.3
FCoV type I	FIPV Black	I. Kiss	34.0
FCoV type II	FIPV DF2	I. Kiss	19.9
FCoV type II	FIPV 79-1146	I. Kiss	38.1
FCoV type II	FECV 79-1683	I. Kiss	21.5
PRCoV	PRCV	I. Kiss	43.9
TGEV	Purdue	I. Kiss	14.0
PEDV	PEDV 7	I. Kiss	29.7
Group 2			
HCoV-OC43	OC43	ATCC	9.8
MHV	MHV-A59	P. Rottier	16.3
BCoV	BCO-44175	A. Kheyar	38.9
BCoV	BCQ-2590	A. Kheyar	18.6
BCoV	BCQ-A130	A. Kheyar	19.5
BCoV	BCQ-4	A. Kheyar	17.6
BCoV	BCQ-3708	A. Kheyar	18.2
Group 3			
IBV	IBV927	I. Kiss	25.6
IBV	H120	I. Kiss	19.2
IBV	M 41	I. Kiss	19.5
IBV	IB 4/91 (793B)	I. Kiss	24.4
IBV	AZRI 5508/95	D. Cavanagh	19.5
IBV	B1648	D. Cavanagh	20.6
IBV	UK/918/67	D. Cavanagh	27.2
IBV	D207	D. Cavanagh	19.3
IBV	Gray 390	D. Cavanagh	34.0
IBV	HV-10	D. Cavanagh	21.1
IBV	7/91	D. Cavanagh	19.8
IBV	Arkansas 99	D. Cavanagh	21.0
PhCoV	PhUK/438/94	D. Cavanagh	27.8
TCoV	176	D. Cavanagh	17.3
SARS-CoV	FFM-ic	M. Niedrig	38.1

HCoV Human coronavirus; *CCV* canine coronavirus; *FCoV* feline coronavirus; *PRCoV* porcine respiratory coronavirus; *TGEV* transmissible gastroenteritis virus; *PEDV* porcine epidemic diarrhea virus; *MHV* murine hepatitis virus; *BCoV* bovine coronavirus; *IBV* infectious bronchitis virus; *PhCoV* Pheasant coronavirus; *TCoV* Turkey coronavirus; *na* not available

their genetic diversity, feline coronaviruses (FCoVs) and canine coronaviruses (CCVs) of both type I and type II were tested. The collection contained bovine coronaviruses (BCoVs) causing distinct pathologies, i.e. winter dysentery (BCQ-2590), adult diarrhea (BCQ-A130), pneumonitis (BCO-44175) and neonatal calf diarrhea (BCQ-4 and BCQ-3708). Field and reference infectious bronchitis viruses (IBVs) of different geographical origins were analyzed together with the IBV vaccine strain H120.

The suitability of the real-time RT-PCR as a diagnostic tool was assessed testing clinical samples previously confirmed positive or negative for coronavirus infection. The assay was applied to 75 samples consisting of faeces, nasal swabs and suspension of mesenteric lymph node. Positive specimens originated from animals naturally infected with FCoV, CCV, or BCoV. The panel also included porcine faeces spiked before RNA extraction with a suspension of transmissible gastroenteritis virus (TGEV) as naturally infected material was not available.

Sample processing and RNA extraction

The clinical samples were diluted 1:100 in TE buffer. RNA was extracted from 140 µl specimen (infected cell supernatant or diluted biological material) using the QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Extracted RNA was eluted in 50 µl of DMPC water and stored at −80 °C.

Primer design

Consensus primers were designed using the program Consort, which identifies regions of conservation (J. Blomberg, description available in [24]). Complete genomic sequences of representative coronaviruses were aligned with Clustal X (1.83) [36] and examined with Consort. After the replicase gene was identified as the most conserved region, all coronavirus ORF1a and ORF1b sequences available in GenBank were retrieved to refine the analysis. The most conserved stretches were found in ORF1b, where one pair of degenerate primers was designed to amplify a fragment of 179 bp (Appendix). The forward primer appeared to be the reversed and modified sequence of primer IN-4 previously published by Ksiazek et al. [20]. The sequence, sense and position of the primers in the genome of SARS-CoV Tor2 (AY274119) are as follows: 11-FW: 5′-TGATGATGSNGTTGTNTGYTAYAA-3′ (+) (nt 15647–15670) and 13-RV: 5′-GCATWGTRTGYTGNGARCARAATTC-3′ (−) (nt 15825–15801).

Real-time RT-PCR

A one-step real-time RT-PCR was developed based on SYBR Green detection. All reactions were performed on the Corbett Research Rotor-Gene Real Time Amplification system (RG-3000, Corbett Research, Mortlake, NSW Australia) using the iScript one-step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, USA). Titration of primers 11-FW and 13-RV was achieved using RNA from strains of CCV, BCoV, IBV and HCoV-229E. Reactions were run using reciprocal combinations of primer concentrations including 0.3, 0.6, 0.7, 0.8 and 0.9 µM. A concentration of 0.7 µM for each primer gave the highest sensitivity, together with a limited formation of primer dimers.

The final procedure was performed as follows: 1 µl of RNA was included in a 25-µl reaction mixture containing SYBR Green RT-PCR reaction mix, 0.7 µM of forward and reverse primers, and 0.5 µl of iScript reverse transcriptase. The reverse transcription was carried out at 50 °C for 40 min, followed by the activation of the hot-start DNA polymerase at 95 °C for 5 min and by 50 cycles in three steps: 94 °C for 40 s, 50 °C for 40 s, and 72 °C for 40 s. The first-derivative melting curve analysis was performed by heating the mixture

to 95 °C for 1 min and then cooling to 55 °C for 45 s and heating back to 95 °C at 0.5 °C increments. Samples were considered positive if both an exponential increase of fluorescence and a coronavirus-specific melting peak were observed.

In vitro transcription of complementary RNA (cRNA) standards

Total RNA from the supernatant of cells infected respectively with TGEV Purdue, BCoV BCQ-2590, SARS-CoV FFM-ic, and IBV D207 was reverse-transcribed into first-strand cDNA. The reaction was carried out at 37 °C for 1 h in a 40-µl volume containing 3 µl of RNA, 40 U M-MLV reverse transcriptase (Invitrogen, Carlsbad, California), First-Strand buffer, 10 mM DTT, 200 µM of each dNTP, 10 U of RNAguard ribonuclease inhibitor (Amersham Biosciences, Uppsala, Sweden) and 50 ng of random hexamers. The forward primer 11-FW was modified with a T7 promoter sequence at its 5′ end (11-FWT7 5′-GGATCCTAATACGACTCACTATAGGGAGGTGATGATGSNGTTGTNTGYTAYAA-3′) [10]. Amplification of cDNA from the four viruses was performed using primers 11-FWT7 and 13-RV. The PCR was carried out in a 50-µl volume containing 5 µl of cDNA, 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, USA), PCR buffer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, and 0.5 µM of each primer. Amplification conditions were 95 °C for 15 min, 5 cycles of 94 °C for 1 min, 40 °C for 1 min and 72 °C for 1 min, 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, and 72 °C for 10 min. The PCR products were purified and quantified spectrophotometrically at 260 nm. cRNA standards were transcribed from 1 µg of PCR products using the MEGAscript T7 kit (Ambion, Cambridgeshire, United Kingdom) according to the manufacturer's instructions. After transcription, 5 U of RNAse-free DNAse was added for 40 min at 37 °C to remove the template DNA. The cRNAs were extracted with acidic phenol/chloroform and precipitated with isopropanol. The cRNA pellets were dissolved in 40 µl of nuclease-free water and quantified spectrophotometrically at 260 nm. Serial ten-fold dilutions of the cRNAs (10^5 to 10^0 copies) were prepared in RNAse-free water including 20 ng/µl of yeast tRNA (Ambion, Huntingdon, United Kingdom) as a carrier. The regression lines between the logarithms of the input amounts of cRNAs and the corresponding mean threshold cycle (Ct) values were calculated using the Rotor-Gene software version 6.0.19 (Corbett Research).

Phylogenetic analysis

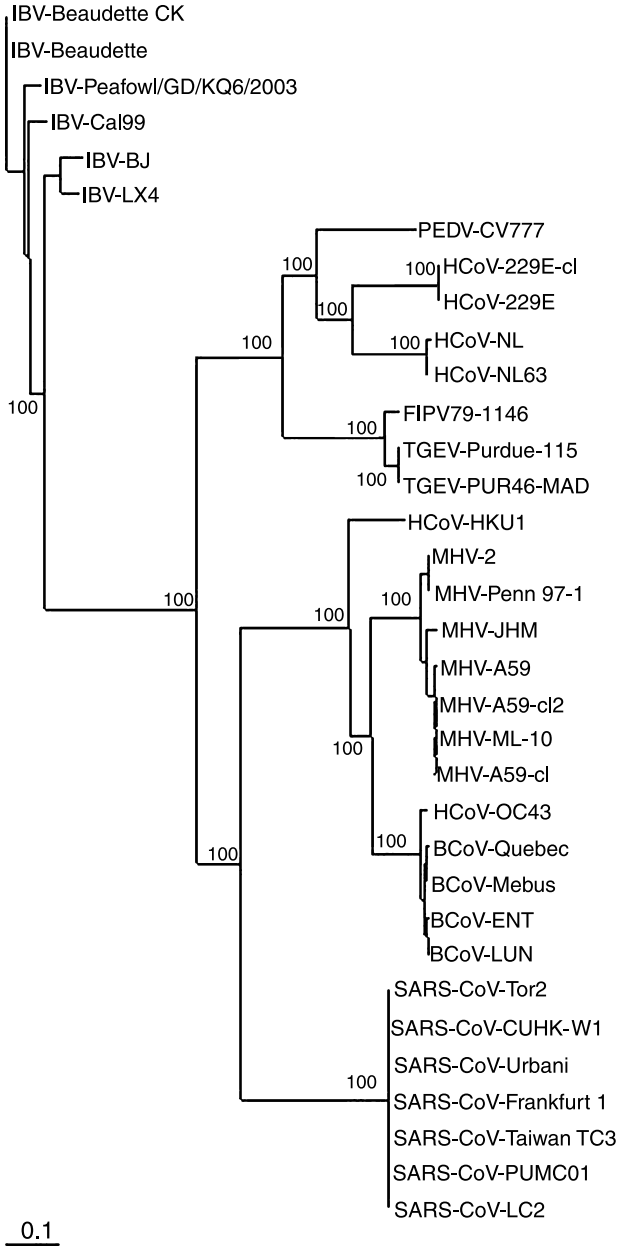
Editing of sequences was done with the BioEdit package (version 7.0.1) [15]. The Clustal X program (version 1.83) [36] was used for sequence alignment. The Maximum-likelihood tree based on ORF1b sequences was constructed by means of the program package TREE-PUZZLE (version 5.2) [34] with 25,000 quartet puzzling steps, the Hasegawa-Kishino-Yano (HKY) model of substitution, and a uniform rate of heterogeneity. Missing parameters were estimated from the data sets.

Results

Evaluation of the real-time RT-PCR on coronavirus strains

Primers 11-FW and 13-RV were designed in conserved stretches of the ORF1b in order to allow a broad reactivity to coronaviruses of the three groups, including SARS-CoV (Fig. 1). The efficacy of the primers was assessed by testing 36 human or animal coronaviruses. All coronavirus RNA samples subjected to real-time RT-PCR presented a specific fluorescence signal and Ct values between 9.8 and 43.9 (Table 1). Amplicons of expected size were visualized by gel electrophoresis.

Sequenced PCR products of TGEV Purdue, porcine respiratory coronavirus (PRCoV), HCoV-NL63, BCoV BCQ-2590, HCoV-OC43, SARS-CoV, and IBV D207 also confirmed the suitability of the method to detect genetically distant coronaviruses (data not shown). In the first-derivative melting curve analysis, the coronavirus amplicons displayed melting temperature (T_m) values between 75.5 and 80.8 °C. Melting peaks for human and animal coronaviruses are shown in Fig. 2A–C. T_m values depend on different factors, including the initial concentration of the template and the size, the GC content, and the sequence of the amplified fragment [29]. In our study, tests on serially diluted RNA and calculation



of theoretical T_m values for genetically distant coronaviruses (data not shown) revealed that the initial template concentration and the genetic variation among viral sequences accounted for the large range of observed T_m values. Despite this broad array, the pattern of melting curve data from the animal coronaviruses did not enable discrimination between the three coronavirus groups or between strains within the same group (Fig. 2B and C). Similarly, differences in T_m values for human coronaviruses are not sufficient for accurate identification of the strains (Fig. 2A).

Sensitivity, reproducibility and specificity of the real-time RT-PCR

The sensitivity of the method was investigated by testing ten-fold serial dilutions of cRNA (10^5–10^0 copies) from TGEV Purdue, BCoV BCQ-2590, SARS-CoV FFM-ic, and IBV D207. All dilutions were tested in triplicate. The amplification plot and first-derivative melting curve analysis were similar for the four viruses and are illustrated with the quantitative analysis of SARS-CoV transcripts in Fig. 3A and B. In all cases, the assay detected down to 10 cRNA copies. The reaction containing 1 cRNA copy generated a fluorescence signal without any coronavirus-specific melting peak (Fig. 3B) and was therefore considered as negative. The nonspecific fluorescence was likely associated with primer dimer formation, which may be observed at low template concentrations [28, 29]. The standard curves displayed a linear relationship between the Ct values and the related numbers of target sequences (Fig. 4).The intra-assay reproducibility was evaluated using triplicate values from the ten-fold serial dilutions. At the highest dilution where cRNA was detected (10 copies per reaction), two out of three replicates were found positive for the four viruses. At the dilution containing 10^2 cRNA copies, 100%

Fig. 1. Phylogenetic tree of representative coronavirus ORF1b nucleotide sequences (nt 13378–21491) included in the alignment generated to design broadly reactive primers. Positions refer to the sequence of SARS-CoV Tor2 (AY274119). The tree was inferred using the maximum-likelihood method in TREE-PUZZLE. Quartet puzzling support values are presented at the branch nodes. Horizontal branches are drawn to a scale of the number of substitutions per site. Distance scale bars indicate a distance of 0.1. Denomination and GenBank accession number of sequences are as follows: feline infectious peritonitis virus (FIPV) 79-1146 (DQ010921), human coronavirus (HCoV)-229E (X69721), HCoV-229E-cl (AF304460), HCoV-NL (AY518894), HCoV-NL63 (AY567487), porcine epidemic diarrhea virus (PEDV)-CV777 (AF353511), transmissible gastroenteritis virus (TGEV)-Purdue-115 (Z34093), TGEV-PUR46-MAD (AJ271965), bovine coronavirus (BCoV)-Quebec (AF220295), BCoV-ENT (AF391541), BCoV-LUN (AF391542), BCoV-Mebus (U00735), HCoV-HKU1 (AY597011), HCoV-OC43 (AY391777), murine hepatitis virus (MHV)-A59 (AF029248), MHV-2 (AF201929), MHV-Penn 97-1 (AF208066), MHV-ML-10 (AF208067), MHV-A59-cl (AY700211), MHV-JHM (M55148), MHV-A59-cl2 (X51939), infectious bronchitis virus (IBV)-Beaudette CK (AJ311317), IBV-BJ (AY319651), IBV-LX4 (AY338732), IBV-Cal99 (AY514485), IBV-Peafowl/GD/KQ6/2003 (AY641576), IBV-Beaudette (M95169), severe acute respiratory syndrome coronavirus (SARS-CoV)-Tor2 (AY274119), SARS-CoV-CUHK-W1 (AY278554), SARS-CoV-Urbani (AY278741), SARS-CoV-Frankfurt 1 (AY291315), SARS-CoV-Taiwan TC3 (AY348314), SARS-CoV-PUMC01 (AY350750), SARS-CoV-LC2 (AY394999)

reproducibility was achieved for all four viruses. The coefficient of variation of Ct values ranged from 0.19 to 1.71% for standard dilutions from 10^2 to 10^5cRNA copies per reaction.

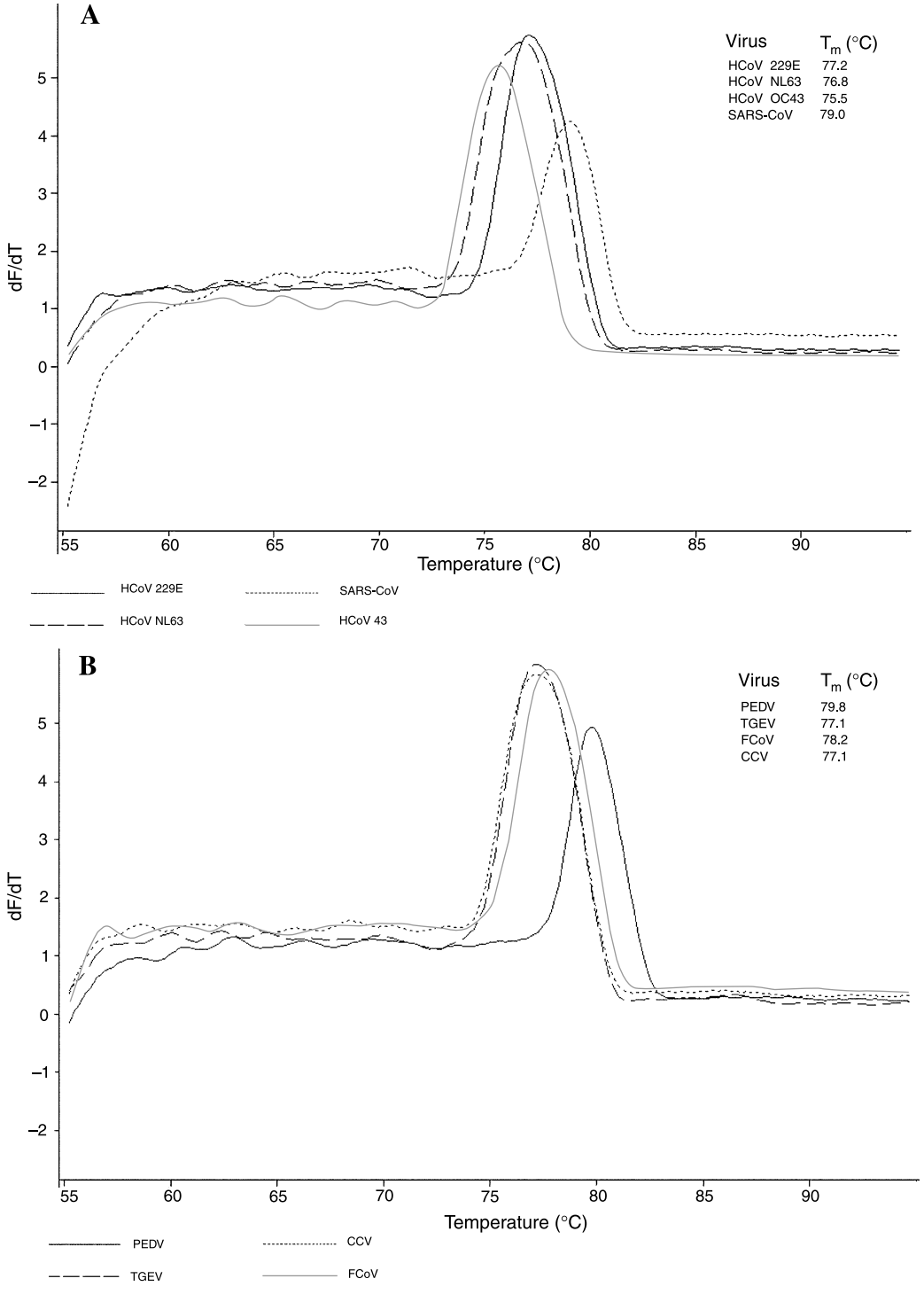


Fig. 2 (*continued*)

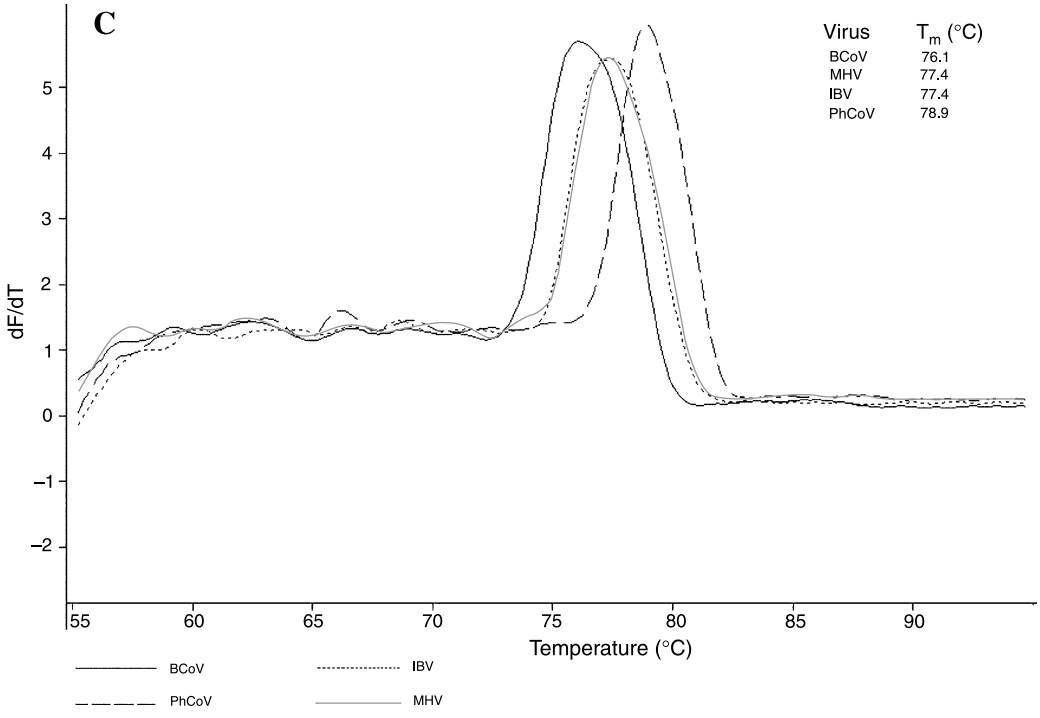


Fig. 2. First-derivative melting curve analysis of the coronavirus real-time reverse transcription-polymerase chain reaction (RT-PCR) using SYBR Green. The curves correspond to the first-derivative of the fluorescence changes (dF/dT) with respect to temperature. Coronavirus PCR products can be distinguished from primer dimers and nonspecific products by melting points included between 75.5 and 80.8 °C. Graphs of human coronaviruses (HCoV) (**A**); group 1 animal coronaviruses: transmissible gastroenteritis virus (TGEV) Purdue, canine coronavirus (CCV) 144/01, feline coronavirus (FCoV) UCD1, and porcine epidemic diarrhea virus (PEDV) 7 (**B**); and groups 2 and 3 animal coronaviruses: bovine coronavirus (BCoV) BCQ-4, MHV A59, infectious bronchitis virus (IBV) 927, and pheasant coronavirus (PhCoV) PhUK/438/94 (**C**)

We examined the specificity of the real-time RT-PCR by testing other RNA viruses, including pathogens of the respiratory or digestive tracts. The panel of viruses contained bovine respiratory syncytial virus, bovine viral diarrhoea virus, swine vesicular disease virus, foot-and-mouth disease virus, vesicular stomatitis virus, hepatitis E, influenza A and B viruses, and equine arteritis virus, another member of the order *Nidovirales*. No positive signal was recorded for any of the strains tested.

Analysis of clinical samples

We next analyzed clinical specimens previously subjected to a coronavirus detection test (Table 2). The panel mainly included faecal samples which might contain PCR inhibitors. The SYBR Green method provided the same positive or negative results as the compared diagnostic assays (Table 2). Positive samples presented a mean Ct value of 29.5 [95% confidence interval (CI) 27.1–31.9; range 14.9–38.2] and a mean T_m of 77.8 °C (95% CI 77.5–78.0; range 77.1–78.9). No primer dimer

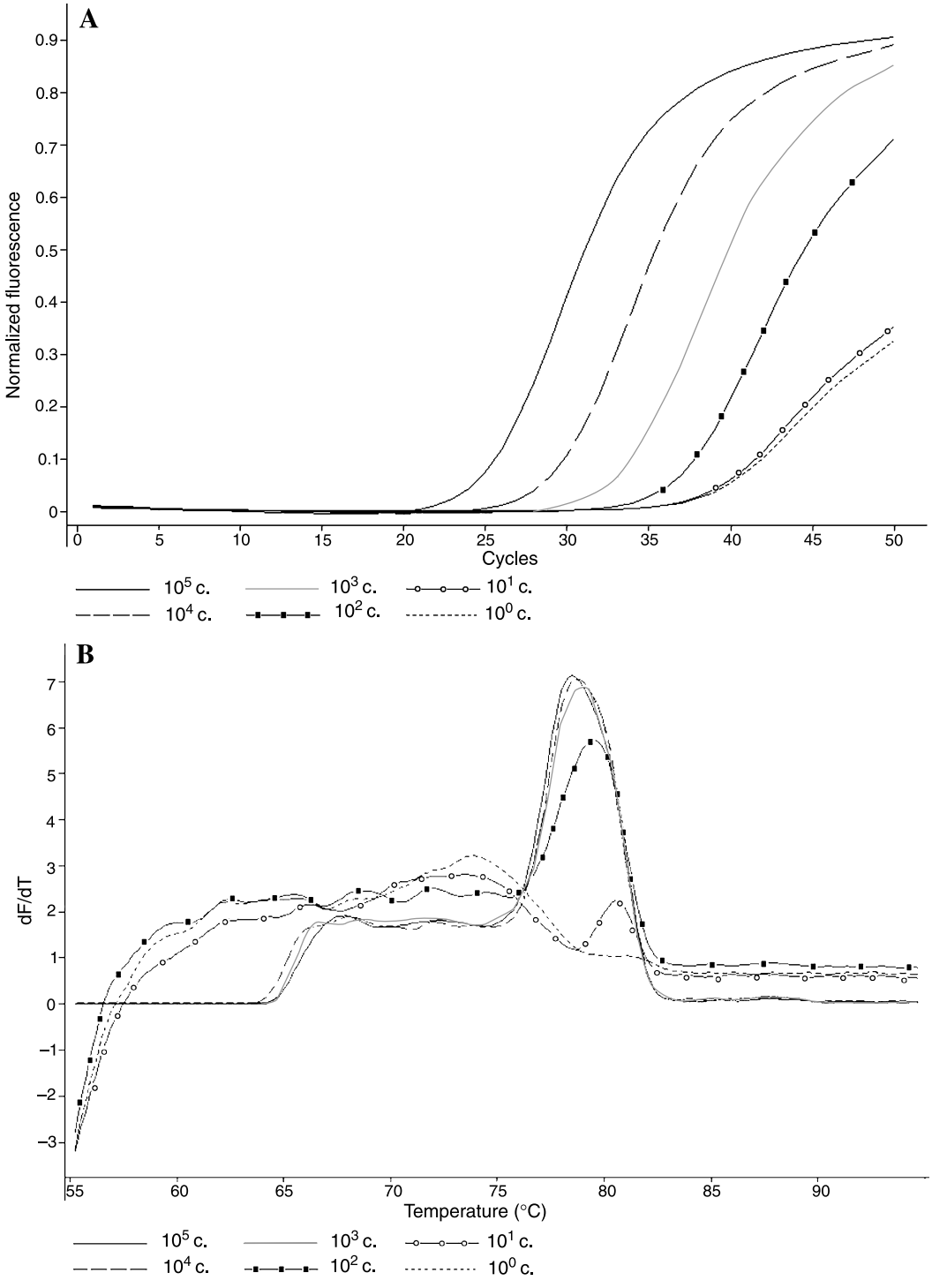


Fig. 3. Amplification plot (**A**) and first-derivative melting curve analysis (**B**) from ten-fold serial dilutions of severe acute respiratory syndrome coronavirus (SARS-CoV) cRNA tested by real-time reverse transcription-polymerase chain reaction. For simplicity, fluorescence data of a single replica of dilutions from 10^5 to 10^0 copies are presented. Normalized fluorescence is background-corrected fluorescence signal

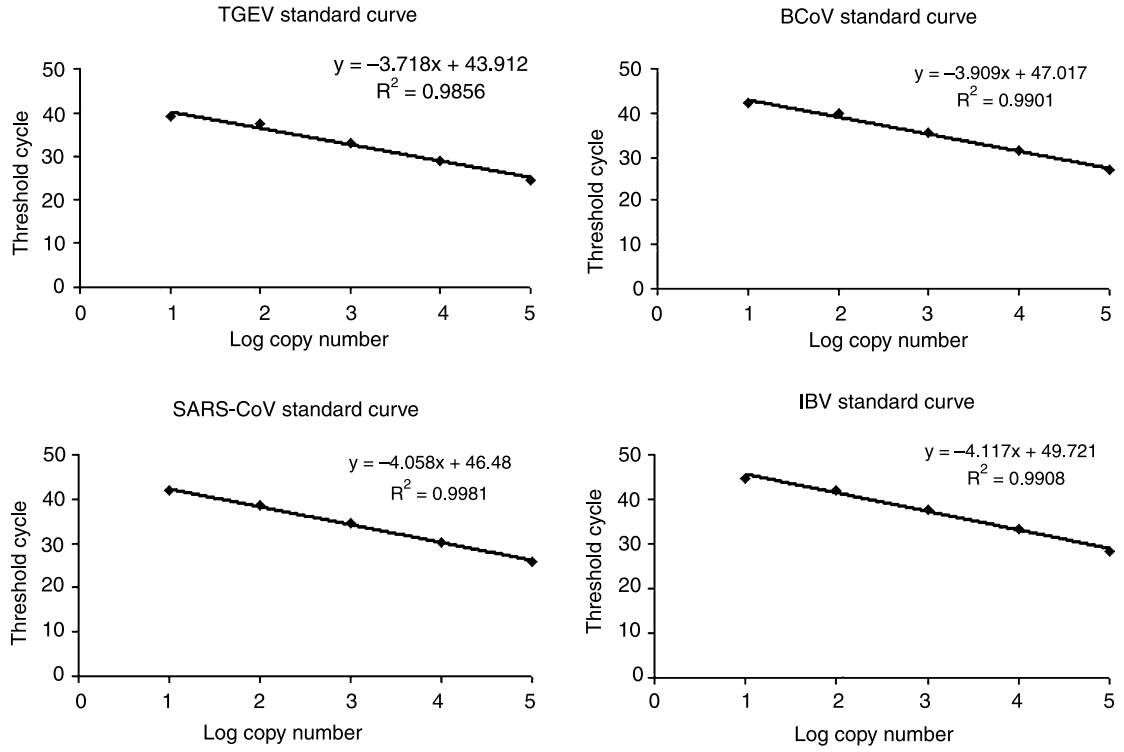


Fig. 4. Standard curves of the real-time reverse transcription-polymerase chain reaction based on serial dilutions of coronavirus cRNA standards. Mean threshold cycle values from three replicates are plotted versus logarithmic concentrations of cRNA copies. For 10 cRNA copies, two out of three replicates were positive. *TGEV* Transmissible gastroenteritis virus; *BCoV* bovine coronavirus; *SARS-CoV* severe acute respiratory syndrome coronavirus; *IBV* infectious bronchitis virus

Table 2. Comparison of SYBR Green and TaqMan real-time reverse transcription-polymerase chain reaction (RT-PCR) assays for the detection of coronavirus infection in clinical samples

Species	Virus	Specimens	Number tested	SYBR Green real-time RT-PCR	Compared diagnostic methods[1]
Canine	CCV	faeces	7	+	+ TaqMan real-time RT-PCR [13]
Canine		faeces	2	−	− TaqMan real-time RT-PCR [13]
Feline	FCoV	mesenteric	1	+	+ TaqMan real-time RT-PCR [13]
Feline		faeces lymph node	4	−	− TaqMan real-time RT-PCR [13]
Porcine[2]	TGEV	faeces	3	+	+ TaqMan real-time RT-PCR [13]
Porcine		faeces	25	−	− TaqMan real-time RT-PCR [13]
Bovine	BCoV	Faeces, nasal swabs	15	+	+ TaqMan real-time RT-PCR [14]
Bovine		Faeces, nasal swabs	18	−	− TaqMan real-time RT-PCR [14]

[1]Tests preliminary performed in the diagnostic or research units of the Department of Virology at the Swedish Veterinary Institute

[2]Samples spiked with a suspension of TGEV before RNA extraction

SG SYBR Green; *CCV* canine coronavirus; *FCoV* feline coronavirus; *TGEV* transmissible gastroenteritis virus; *BCoV* bovine coronavirus

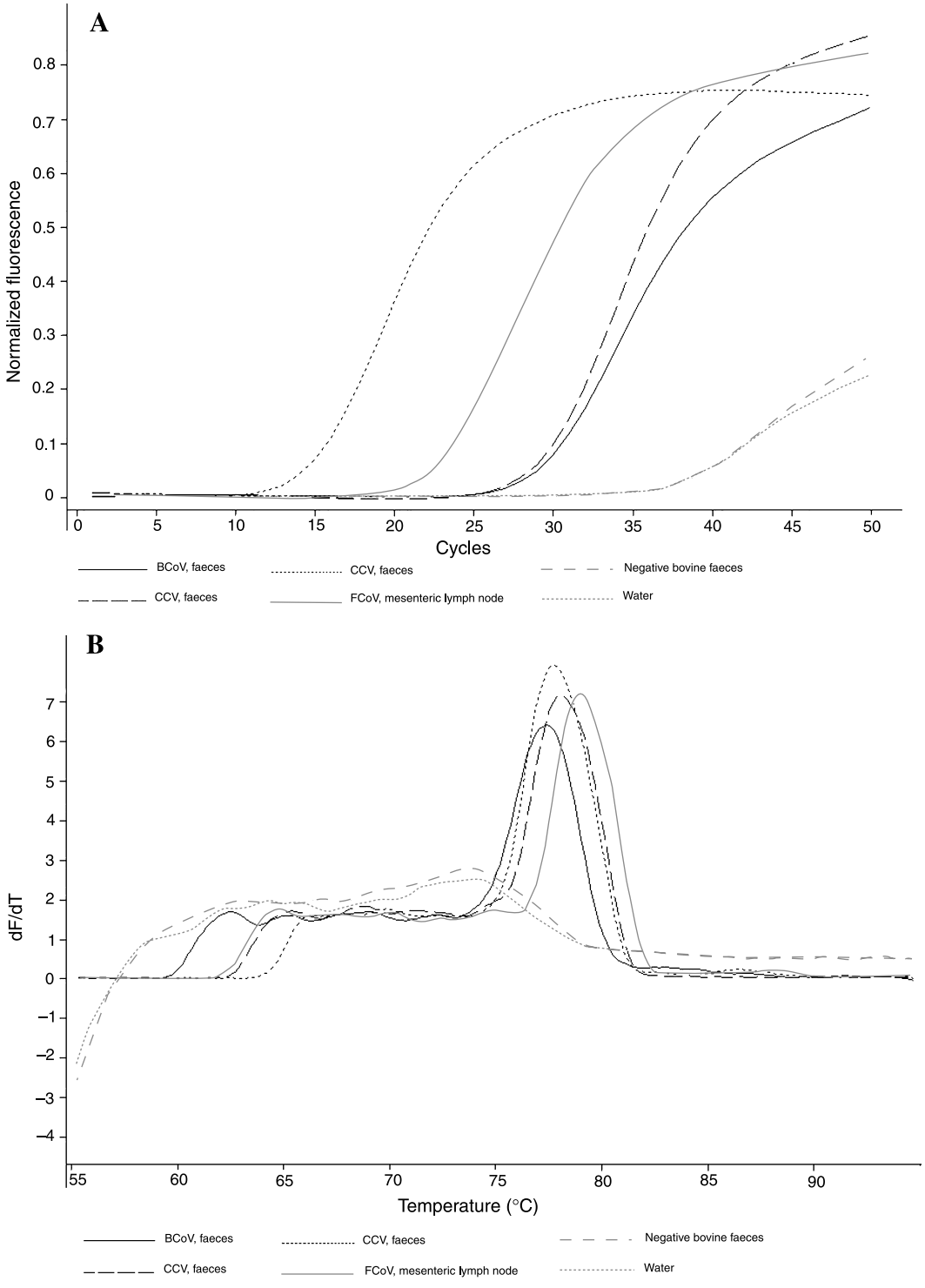


Fig. 5. Detection of coronaviruses in clinical samples by real-time reverse transcription-polymerase chain reaction. Amplification plot (**A**) and first-derivative melting curves (**B**) from canine coronavirus (CCV), feline coronavirus (FCoV), and bovine coronavirus (BCoV) infected specimens and from negative controls

formation or spurious product was observed in positive samples as determined by the first-derivative melting curve analysis. The optic graph and melting peaks of FCoV, CCV and BCoV amplicons are shown in Fig. 5A and B, respectively. In negative specimens and negative controls, primer dimers produced fluorescence and a small melting peak at 74 °C, easily distinguishable from the coronavirus-specific peaks (Fig. 5A and B). To assess the sensitivity of the method on clinical material, we tested ten-fold serial dilutions of RNA extracted from two faecal samples of dogs naturally infected with CCV. The assay could detect viral RNA down to dilutions of $1{:}10^5$ and $1{:}10^3$, respectively (data not shown).

To further investigate the adequacy of the method in the presence of potential PCR inhibitors, RNA extracts from stools of eight wild ruminants were spiked with BCoV RNA. A specific fluorescence signal was recorded in all samples tested. Whereas the BCoV positive control displayed a Ct value of 17.6, the specimens spiked with the same amount of BCoV RNA presented a mean Ct value of 18.7 (95% CI 18.2–19.1; range 17.8–19.7).

Discussion

Pancoronavirus detection methods are needed both in human and in veterinary medicine. Although often overlooked in the past, coronaviruses revealed their importance in public health by the recognition of SARS-CoV, HCoV-NL63 and CoV-HKU1 as causative agents of severe respiratory diseases [6, 9, 20, 26, 38, 41]. Additional pathogenic human coronaviruses, including enteric variants, might still be identified if investigated with proper diagnostic tools. Coronaviral disease is also a veterinary problem. The high complexity and genetic diversity of coronaviruses and the economic losses the viruses generate in breeding animals justify efforts to develop broad-spectrum detection techniques. In addition, such assays would be relevant to identify animal reservoirs of new pathogenic coronaviruses.

Setting up a generic RT-PCR is tedious due to the high genetic variation between and within the three coronavirus groups. In this study, we developed a one-step real-time RT-PCR targeting a conserved region of the coronavirus ORF1b. Based on SYBR Green chemistry, degenerate primers, and a low annealing temperature, the assay enabled the detection of 36 different strains of human and animal coronaviruses (Table 1). The method proved to be suitable for diagnostic purposes, showing high sensitivity and specificity when applied to biological samples from different animal species. Positive fluorescence signals in all faecal samples naturally infected or spiked with coronaviruses suggest that the real-time RT-PCR is robust, even if potential PCR inhibitors are present. The detection of HCoV-OC43-like or HCoV-NL63 sequences in eight out of 85 sputum specimens of human origin provides further evidence of the efficacy of the technique on clinical material (Mohamed et al., manuscript in preparation).

Based on serially diluted cRNA standards, the SYBR Green real-time RT-PCR detected down to 10 cRNA copies from TGEV, BCoV, SARS-CoV, and IBV. The sensitivity of our method is similar to the sensitivity of other real-time PCR assays based on the ORF1b for the specific detection of SARS-CoV, including

the commercial SARS-CoV Quantification kit (Roche, Penzberg, Germany) [6, 7, 19, 22, 25, 27]. The RealArt HPA coronavirus LC kit (Artus, Hamburg, Germany) nevertheless seems to be more sensitive, with a detection level down to 0.5–1.5 SARS-CoV RNA copy per reaction [4]. Targeting the N gene has been postulated to be a better choice to improve the sensitivity of the PCR as this gene is supposed to have the most abundant copy number during viral replication [35]. Indeed, some protocols using N primers were found to be more sensitive than those based on the replicase gene for detecting SARS-CoV [17, 21]. However, considering the lower degree of conservation of the N gene, a PCR targeting this region of the genome would be much less likely to detect the different coronavirus clusters equally.

The specificity of our test was demonstrated by the absence of positive reaction with other RNA viruses and with clinical samples confirmed negative for coronavirus infection. The first-derivative melting curve analysis enabled specific coronavirus sequence amplification to be discriminated from primer dimers or potential nonspecific products. The ability to distinguish different coronavirus strains from each other depends on the pattern of their melting peaks. Because of the variation of T_m values between and within the three coronavirus groups, sequencing of the PCR products is a better alternative for accurate identification of viruses detected in clinical samples.

Primer dimers were present in negative samples or when the amount of coronavirus RNA was low. This phenomenon is likely related to the low annealing temperature and the high degeneracy and high concentration of the primers. The primer dimer formation makes our method unsuitable for strict quantification purposes, despite the linear relationship between Ct values and related cRNA copies. An optical read step at a temperature higher than the T_m of primer dimers (74 °C) could have significantly reduced the detection of nonspecific fluorescence. However, such a modification of the optical read was not applied due to the close range of the coronavirus T_m values (75.5–80.8 °C). Alternatively, the ratio of product melting in the expected range to total product could be used to correct for nonspecific amplification [29]. Nevertheless, the possible competition between specific amplification and primer dimer formation at low template concentration might not be avoided. In addition, the melting peak associated with primer dimers might also hamper the detection of a coronavirus that would present a T_m value lower than those recorded for the strains tested in this study.

Compared with TaqMan probes, SYBR Green is not sensitive to mutations in the amplified sequence and is a lower-cost alternative. This chemistry is of particular interest when targeting a broad range of genetically distant viruses. The iScript kit used in this study limited the variability of the reagents and was convenient for the optimization and standardization of the reaction protocol. When tested on positive clinical samples, the iScript kit also proved to be more sensitive than the EZ rTth RNA PCR kit using SYBR Green I (Molecular Probes, Eugene, USA) diluted 1:100,000 in TE buffer (data not shown).

To our knowledge, this is the first report of a pancoronavirus method adapted to real-time format and tested on a wide range of viral strains and clinical samples. The method presents a potential utility as a diagnostic tool in clinical laboratories or to detect still uncharacterized and/or newly emerging coronaviruses.

Appendix

Result of the conservation analysis from ConSort in the coronavirus ORF1b segment selected for amplification. All nucleotide variants for each position are displayed, and a consensus sequence is provided. The positions of primers 11-FW and 13-RV are underlined. Numbers refer to positions in the alignment submitted to ConSort (see Materials and methods).

```
                14619                                          11-FW   14678
                 |         |         |         |         |         |
Consensus       WYTWYDVDTWYYTDHRDAARMAYTTYWSHWTGATGATYYTNWSYGAYGAYRSNGTTGTBT

                ATTATGGTTATTTAAGTAAGCATTTTTCTATGATGATTTTGTCTGATGATGGTGTTGTGT
                TC TCAAA TCC GCAA  AA C  CAGAT       CC TAGC  C  CACC     T
                     TCG     TT G          C           A          A     C
                                                       C          G


                14679                                                  14738
                 |         |         |         |         |         |
                GYTAYRAYWVDRMNYWWGCNDVNHWDGGKYWHRTHGCDRRYMTWWVBRVHTTYMRRSMNR

                GTTATAACAATGATTATGCGTCACAAGGTTATATAGCTGATATTAGTGCTTTTAAAGCAG
                 C  CG TTCAACCCTA  TGACATT  GCTAG T  AAGCC ATAGAAC  CCGGCAGA
                        GG  A     AAGTT G     C  C  G       CC GA        C
                            G     C  G                                   T


                14739                                                  14798
                 |         |         |         |         |         |
                YWYTNTAYTAYCARAAYRRYGTBTTYATGWSHRMNKCHAARTGTTGGRYNGARVMWGAYH

                TTTTGTATTATCAAAATAATGTCTTTATGTCTGAATCTAAATGTTGGGTTGAACCAGACA
                CAC A  C  C  G  CGGC  G  C   AGAACTG C  G      ACA  GAAT  TC
                    T                T        C  G  A          G   G     T
                    C                         C              C


                14799                  13-RV                    14858
                 |         |         |         |         |         |
                THDVHRWDGGHCCDCAYGARTTYTGYTCNCARCAYACWWTGCWDRTNRWNVDDVVDRRBG

                TAAATAAAGGACCTCATGAATTTTGTTCACAGCATACAATGCTAGTTGAGGAGGATGATG
                 TGCAGTT  T  A  C  G  C  C  T  A  C  TT   AGA AATAATTAGAAGG
                 CTGC  G  C  G            G             T  G  TCGACCG  C
                                          C                C  C
```

Acknowledgments

We thank L. van der Hoek (Academic Medical Center, University of Amsterdam, The Netherlands), I. Kiss (Veterinary Institute of Debrecen, Hungary), C. Buonavoglia (Faculty of Veterinary Medicine, University of Bari, Italy), P. Rottier (Department of

Infectious Diseases and Immunology, University of Utrecht, The Netherlands), D. Cavanagh (Institute for Animal Health, Compton, United Kingdom) and A. Kheyar (Armand Frappier Institute, Laval, Canada) for contribution to the collection of coronavirus strains. SARS-CoV standard was kindly provided by M. Niedrig, Robert Koch-Institut, Berlin. We acknowledge M. Hakhverdyan, S. Alenius, M. Tråvén and J. Wensman for providing clinical samples and for useful advice. The work was supported and financed by project 22.1/2003–1059 of the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS).

References

1. Brian DA, Baric RS (2005) Coronavirus genome structure and replication. Curr Top Microbiol Immunol 287: 1–30
2. Callison SA, Hilt DA, Jackwood MW (2005) Rapid differentiation of avian infectious bronchitis virus isolates by sample to residual ratio quantitation using real-time reverse transcriptase-polymerase chain reaction. J Virol Methods 124: 183–190
3. Chen R, Huang W, Lin Z, Zhou Z, Yu H, Zhu D (2004) Development of a novel real-time RT-PCR assay with LUX primer for the detection of swine transmissible gastroenteritis virus. J Virol Methods 122: 57–61
4. Chui L, Drebot M, Andonov A, Petrich A, Glushek M, Mahony J (2005) Comparison of 9 different PCR primers for the rapid detection of severe acute respiratory syndrome coronavirus using 2 RNA extraction methods. Diagn Microbiol Infect Dis 53: 47–55
5. Drosten C, Chiu LL, Panning M, Leong HN, Preiser W, Tam JS, Gunther S, Kramme S, Emmerich P, Ng WL, Schmitz H, Koay ES (2004) Evaluation of advanced reverse transcription-PCR assays and an alternative PCR target region for detection of severe acute respiratory syndrome-associated coronavirus. J Clin Microbiol 42: 2043–2047
6. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnikova L, Fouchier RA, Berger A, Burguiere AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Muller S, Rickerts V, Sturmer M, Vieth S, Klenk HD, Osterhaus AD, Schmitz H, Doerr HW (2003) Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med 348: 1967–1976
7. Emery SL, Erdman DD, Bowen MD, Newton BR, Winchell JM, Meyer RF, Tong S, Cook BT, Holloway BP, McCaustland KA, Rota PA, Bankamp B, Lowe LE, Ksiazek TG, Bellini WJ, Anderson LJ (2004) Real-time reverse transcription-polymerase chain reaction assay for SARS-associated coronavirus. Emerg Infect Dis 10: 311–316
8. Enjuanes L, Brian D, Cavanagh D, Holmes K, Lai MMC, Laude H, Masters P, Rottier PJM, Siddell SG, Spaan WJM, Taguchi F, Talbot P (2000) Coronaviridae. In: van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR, Wickner RB (eds) Virus Taxonomy, Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, pp 835–849
9. Fouchier RA, Hartwig NG, Bestebroer TM, Niemeyer B, de Jong JC, Simon JH, Osterhaus AD (2004) A previously undescribed coronavirus associated with respiratory disease in humans. Proc Natl Acad Sci USA 101: 6212–6216
10. Fronhoffs S, Totzke G, Stier S, Wernert N, Rothe M, Brüning T, Koch B, Sachinidis A, Vetter H, Ko Y (2002) A method for the rapid construction of cRNA standard curves in quantitative real-time reverse transcription-polymerase chain reaction. Mol Cell Probes 16: 99–110

11. Gagneur A, Sizun J, Vallet S, Legr MC, Picard B, Talbot PJ (2002) Coronavirus-related nosocomial viral respiratory infections in a neonatal and paediatric intensive care unit: a prospective study. J Hosp Infect 51: 59–64
12. Gorbalenya AE, Snijder EJ, Spaan WJ (2004) Severe acute respiratory syndrome coronavirus phylogeny: toward consensus. J Virol 78: 7863–7866
13. Gut M, Leutenegger CM, Huder JB, Pedersen NC, Lutz H (1999) One-tube fluorogenic reverse transcription-polymerase chain reaction for the quantitation of feline coronaviruses. J Virol Methods 77: 37–46
14. Hakhverdyan M, Willman S, Thorén P, Larsen L-E, Alenius S, Belak S (2003) Detection of bovine respiratory syncytial virus and bovine coronavirus using multiplex real-time PCR. In: European Society for Veterinary Virology, Sixth International Congress of Veterinary Virology, St Malo, France, p 60
15. Hall TA (1999) BioEdit: a user-friendly biological sequence alignment and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95–98
16. Hu W, Bai B, Hu Z, Chen Z, An X, Tang L, Yang J, Wang H (2005) Development and evaluation of a multitarget real-time Taqman reverse transcription-PCR assay for detection of the severe acute respiratory syndrome-associated coronavirus and surveillance for an apparently related coronavirus found in masked palm civets. J Clin Microbiol 43: 2041–2046
17. Hui RK, Zeng F, Chan CM, Yuen KY, Peiris JS, Leung FC (2004) Reverse transcriptase PCR diagnostic assay for the coronavirus associated with severe acute respiratory syndrome. J Clin Microbiol 42: 1994–1999
18. Jackwood MW, Hilt DA, Callison SA (2003) Detection of infectious bronchitis virus by real-time reverse transcriptase-polymerase chain reaction and identification of a quasispecies in the Beaudette strain. Avian Dis 47: 718–724
19. Keyaerts E, Vijgen L, Maes P, Duson G, Neyts J, Van Ranst M (2006) Viral load quantitation of SARS-coronavirus RNA using a one-step real-time RT-PCR. Int J Infect Dis 10: 32–37
20. Ksiazek TG, Erdman D, Goldsmith CS, Zaki SR, Peret T, Emery S, Tong S, Urbani C, Comer JA, Lim W, Rollin PE, Dowell SF, Ling AE, Humphrey CD, Shieh WJ, Guarner J, Paddock CD, Rota P, Fields B, DeRisi J, Yang JY, Cox N, Hughes JM, LeDuc JW, Bellini WJ, Anderson LJ (2003) A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 348: 1953–1966
21. Kuiken T, Fouchier RA, Schutten M, Rimmelzwaan GF, van Amerongen G, van Riel D, Laman JD, de Jong T, van Doornum G, Lim W, Ling AE, Chan PK, Tam JS, Zambon MC, Gopal R, Drosten C, van der Werf S, Escriou N, Manuguerra JC, Stohr K, Peiris JS, Osterhaus AD (2003) Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. Lancet 362: 263–270
22. Mahony JB, Petrich A, Louie L, Song X, Chong S, Smieja M, Chernesky M, Loeb M, Richardson S (2004) Performance and Cost evaluation of one commercial and six in-house conventional and real-time reverse transcription-PCR assays for detection of severe acute respiratory syndrome coronavirus. J Clin Microbiol 42: 1471–1476
23. Moes E, Vijgen L, Keyaerts E, Zlateva K, Li S, Maes P, Pyrc K, Berkhout B, van der Hoek L, Van Ranst M (2005) A novel pancoronavirus RT-PCR assay: frequent detection of human coronavirus NL63 in children hospitalized with respiratory tract infections in Belgium. BMC Infect Dis 5: 6
24. Mohamed N, Belak S, Hedlund KO, Blomberg J (2006) Experience from the development of a diagnostic single tube real-time PCR for human caliciviruses, *Norovirus* genogroups I and II. J Virol Methods 132: 69–76

25. Nitsche A, Schweiger B, Ellerbrok H, Niedrig M, Pauli G (2004) SARS coronavirus detection. Emerg Infect Dis 10: 1300–1303
26. Peiris JS, Lai ST, Poon LL, Guan Y, Yam LY, Lim W, Nicholls J, Yee WK, Yan WW, Cheung MT, Cheng VC, Chan KH, Tsang DN, Yung RW, Ng TK, Yuen KY (2003) Coronavirus as a possible cause of severe acute respiratory syndrome. Lancet 361: 1319–1325
27. Poon LL, Wong OK, Chan KH, Luk W, Yuen KY, Peiris JS, Guan Y (2003) Rapid diagnosis of a coronavirus associated with severe acute respiratory syndrome (SARS). Clin Chem 49: 953–955
28. Richards GP, Watson MA, Fankhauser RL, Monroe SS (2004) Genogroup I and II noroviruses detected in stool samples by real-time reverse transcription-PCR using highly degenerate universal primers. Appl Environ Microbiol 70: 7179–7184
29. Ririe KM, Rasmussen RP, Wittwer CT (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal Biochem 245: 154–160
30. Saif LJ (2004) Animal coronaviruses: what can they teach us about the severe acute respiratory syndrome? Rev Sci Tech 23: 643–660
31. Sampath R, Hofstadler SA, Blyn LB, Eshoo MW, Hall TA, Massire C, Levene HM, Hannis JC, Harrell PM, Neuman B, Buchmeier MJ, Jiang Y, Ranken R, Drader JJ, Samant V, Griffey RH, McNeil JA, Crooke ST, Ecker DJ (2005) Rapid identification of emerging pathogens: coronavirus. Emerg Infect Dis 11: 373–379
32. Spackman E, Kapczynski D, Sellers H (2005) Multiplex real-time reverse transcription-polymerase chain reaction for the detection of three viruses associated with poult enteritis complex: turkey astrovirus, turkey coronavirus, and turkey reovirus. Avian Dis 49: 86–91
33. Stephensen CB, Casebolt DB, Gangopadhyay NN (1999) Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronaviruses and development of a consensus polymerase chain reaction assay. Virus Res 60: 181–189
34. Strimmer K, Von Haeseler A (1996) Quartet puzzling: a quartet maximum-likelihood method for reconstructing TREE topologies. Mol Biol Evol 13: 964–969
35. Thiel V, Ivanov KA, Putics A, Hertzig T, Schelle B, Bayer S, Weissbrich B, Snijder EJ, Rabenau H, Doerr HW, Gorbalenya AE, Ziebuhr J (2003) Mechanisms and enzymes involved in SARS coronavirus genome expression. J Gen Virol 84: 2305–2315
36. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24: 4876–4882
37. Vabret A, Mourez T, Gouarin S, Petitjean J, Freymuth F (2003) An outbreak of coronavirus OC43 respiratory infection in Normandy, France. Clin Infect Dis 36: 985–989
38. van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, Wertheim-van Dillen PM, Kaandorp J, Spaargaren J, Berkhout B (2004) Identification of a new human coronavirus. Nat Med 10: 368–373
39. Vijgen L, Keyaerts E, Moes E, Maes P, Duson G, Van Ranst M (2005) Development of one-step, real-time, quantitative reverse transcriptase PCR assays for absolute quantitation of human coronaviruses OC43 and 229E. J Clin Microbiol 43: 5452–5456
40. WHO (2003) Cumulative Number of Reported Probable Cases of SARS. From: 1 Nov 2002 to: 11 July 2003, http://www.who.int/csr/sars/country/2003_07_11/en/
41. Woo PC, Lau SK, Chu CM, Chan KH, Tsoi HW, Huang Y, Wong BH, Poon RW, Cai JJ, Luk WK, Poon LL, Wong SS, Guan Y, Peiris JS, Yuen KY (2005) Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J Virol 79: 884–895

Author's address: S. Escutenaire, Department of Virology, National Veterinary Institute, Ullsväg 2B, 75189 Uppsala, Sweden; e-mail: sophie.escutenaire@hotmail.com