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

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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-
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′-TGATGATGNGTTGTNTGYTAY AA-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′-GGATCCTAAT ACCGACTCAGTGTGAAGGAGTGATGATGSNGTTGTNTGYTAYAA-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 MgCl2, 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 10^6 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%
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 $10^5$ cRNA copies per reaction.

**Fig. 2 (continued)**

| Virus          | $T_m$ (°C) |
|----------------|------------|
| HCoV 229E      | 77.2       |
| HCoV NL63      | 76.0       |
| HCoV OCA3      | 75.5       |
| SARS-CoV       | 79.0       |

| Virus          | $T_m$ (°C) |
|----------------|------------|
| PEDV           | 79.8       |
| TGEV           | 77.1       |
| FGaV           | 78.2       |
| CCV            | 77.1       |
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).

| Consensus | WYTWDVDTWTYDTYDDAARMAYTTYWSHTGATGATYTNW | YGAYGAYRSNGTGTGT | 11-FW | WYTWDVDTWTYDTYDDAARMAYTTYWSHTGATGATYTNW | YGAYGAYRSNGTGTGT |
|-----------|------------------------------------------|------------------|-------|------------------------------------------|------------------|
| ATTATGTTATTTAAAGCAGCTTTTCTATGATGATTTGTC | TGATGATGTTGTGTTG | TCAAA | TCC | CCAGAT | TGAC | C | CACC | T |
| TCG | TT | G | C | A | A | C | C | G |

| 14619 | 11-FW | 14678 |
|-------|-------|-------|
| GYTA YRA | WVD MNY WYG CNV NH WDD GKY WH RTG | CDR Y M T W V B R V H T Y M R S |

| GTTATAAC | CAGATTTATAGTCACGACAGGTTATATATGATGATTAGTGTGTTTTAAAGCAG | C | CG | TTCAACCTTA | TGACAT | GCTAG | T | AAGCC | ATAGAAC | CCGGCAGA |
| G | A | AAGTT | G | C | C | GG | C | GA | C | G |

| 14739 | 14798 |
|-------|-------|
| YWYT TAYTAYCAARAAYRYYRTG BTYYATGWSHRMNKCHAAARTGTTGGRYNGARVMW GAYH |

| TTTTGATTTATCAAATAATATGCTTTATGCTGAATCTAAATATGTTGGTTGAGACCAGACA | CAC | A | C | C | CGGC | G | C | AGACTG | C | G | ACA | GAAT | TC |
| T | T | C | G | A | G | G | T | C | C |

| 14799 | 14858 |
|-------|-------|
| TDVHRWDDGHCCDCA YGARTTYTGYT-CNCA CARCAYACWWTG | WDRTRNRNVDDVVDRRB |

| TAAAATAGACCTCATGAAATTTGTTAACAGCATAATGCTAGTTGAGGAGGAGGTGATG | TGCAGTT | T | A | C | C | C | T | A | C | T | AGA | ATAAATTTAGAAAG |
| C2GC | G | C | G | G | T | G | TCGACCG | C | C |

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