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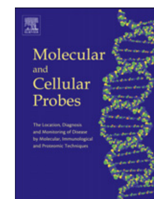

# Development of a SYBR Green I based real-time RT-PCR assay for detection and quantification of bovine coronavirus

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ABSTRACT

A novel two-step, SYBR Green I based real-time RT-PCR assay was developed for detection and quantification of BCoV using ABI PRISM 7500 sequence detection system. The assay was carried out using two sets of primers designed to amplify highly conserved sequences of the nucleocapsid gene of BCoV and the internal control, bovine glyceraldehyde-3-phosphate dehydrogenase, RNA. Specific identification of both targets was elucidated by melt curve analysis, in which the BCoV amplified product generated a melt peak at $78.35 \pm 0.26$ °C and the internal control RNA at $82.54 \pm 0.32$ °C. The assay was highly specific since all negative controls and other viruses of clinical and structural relevance failed to develop any positive results. The detection limit of the reaction was $10^3$ plasmid copies and $1.17 \times 10^{-3}$ $TCID_{50}$ of the tissue culture propagated virus. Standard deviation and coefficient of variation was low for both intra-assay and inter-assay variability. The assay performance on field samples was evaluated on 103 (68 fecal and 35 nasal) swab specimens and compared with the conventional RT-PCR assay. The results of both assays matched for the diagnosis of 65 fecal and 33 nasal samples. However, three fecal and two nasal samples tested negative in gel-based assay were positive for the real-time RT-PCR. The robustness and a high-throughput performance of the developed assay make it a powerful tool in diagnostic applications and in BCoV research.

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## 1. Introduction

Bovine coronavirus is the second major cause of diarrhea that affects calves during the first 3 weeks of life [1]. The virus also infects the bovine respiratory tract and it has been associated with winter dysentery in adult cattle [2,3]. Disease outcomes' induced by BCoV are responsible for significant economical losses to beef and dairy industries worldwide. These losses are not only the figure of mortalities, which may reach 80% in some complicated cases, but also reflect the veterinary costs, medications and poor productivity of affected animals [4].

BCoV is a member of the antigenic group 2 of genus *Coronavirus* that belongs to family *Coronaviridae* within the order *Nidovirales* [5]. The virion possesses a single stranded, non-segmented, linear RNA genome of positive polarity that is capped, polyadenylated and infectious [6]. The viral genome is associated with the nucleoprotein (N) to form a long, flexible and helical nucleocapsid. The nucleocapsid is wrapped within a lipoprotein envelope that carries four structural proteins; the integral membrane glycoprotein (M); the spike glycoprotein (S); the hemagglutinin–esterase glycoprotein (HE) and the small envelope protein (E) [7].

Although several methods have been used for diagnosis of BCoV infections, there is no "gold standard" test exists. Virus isolation with the G clone of HRT-18 cells has long been considered the most sensitive tool available but this procedure was difficult, time-consuming and not practical for high-throughput screening of samples [8]. Molecular assays such as RT-PCR offered a suitable alternative to virus isolation since they are faster, simpler yet maintain the higher performance and accuracy required for conclusive rapid diagnosis of field outbreaks [9,10].

The advent of real-time PCR methods has further improved the significant benefits of RT-PCR. Compared to conventional gel-based PCR assays, real-time PCR has many advantages including rapidity, higher sensitivity, specificity and throughput, lower hand-on-time, minimal contamination rate, quantitative measurement, automation, and easy standardization [11]. All real-time amplification

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methods depend on the detection and quantification of a fluorescent reporter molecule whose signal increases in proportion to the generated amplicon. The simplest and most cost-effective method involves the use of DNA intercalating dye such as SYBR Green I as a fluorescent reporter. However, the major disadvantage is that the dye molecules may bind to any double stranded DNA in the reaction including non-specific products and primer-dimmers, which requires melt curve analysis to control the specificity of the reaction [12]. A single report described the use of SYBR Green I for detection of 32 animal coronaviruses including BCoV using degenerate primers [13]. This test could be used only for detection of new, uncharacterized coronaviruses rather than for specific identification of BCoV.

In this report, a real-time RT-PCR system using SYBR Green I and melt curve analysis was developed for detection and quantification of BCoV in clinical samples. The assay performance was verified by the evaluation of specificity, sensitivity, reproducibility and dynamic range.

## 2. Materials and methods

### 2.1. Viruses

The different viruses used in this study are listed in Table 1. BCoV reference (Mebus) strain was used throughout this study to standardize the real-time RT-PCR assay. Several enteric and respiratory bovine viruses were included to test the specificity of the developed assay. A single coronavirus (avian infectious bronchitis virus) was used for evaluation of the cross reactivity. Other viruses were included as negative controls.

### 2.2. Clinical specimens

A total of 103 bovine clinical samples were collected from affected cases with respiratory and/or enteric manifestations. These samples included 68 fecal and 35 nasal swabs. Swabs from BCoV-free calves were also included to serve as negative controls and matrices for sensitivity testing. Immediately after collection, swabs were placed in 1 ml viral transport medium consisting of Minimal essential medium (MEM), 500 U penicillin and 500 μg streptomycin; transported on dry ice and stored directly at −80 °C until processing. For sample preparation, all swabs were discarded after pulse-vortexing for 15 s; sample suspension was diluted 1:10 in MEM and clarified by centrifugation at 5000 rpm for 15 min at 4 °C. The supernatant was transferred to sterile vials where aliquots of 140 μl were used for viral RNA extraction and the rest was stored at −80 °C.

### 2.3. Primer design and synthesis

The N gene sequences from 28 BCoV known isolates worldwide were aligned with the Clustal W method available as part of the Megalign program in the Lasergene sequence analysis package version 3.18 (DNAStar, Madison, WI, USA). A specific oligonucleotide primer set was designed with the Primer3 program (Agilent Technologies, Santa Clara, CA, USA) for use in real-time PCR to amplify as many as BCoV strains as theoretically possible. No sequence homology revealed using these primers with group 1 and 3 coronaviruses, however, cross reactivity with members of group 2 coronaviruses is expected. Previously described primers for amplification of the entire N gene were used in construction of the quantification standards (Amer et al., 2008). Internal control primers were designed on the basis of highly conserved sequences in the bovine glyceraldehyde-3-phosphate dehydrogenase (BGAPDH) gene for use in validation of RNA extraction, cDNA synthesis and qPCR steps. All primers were synthesized at Metabion (Martinsried, Germany). Details of primers sequences and locations are listed in Table 2.

### 2.4. Viral RNA extraction and cDNA synthesis

Viral RNA was extracted from the different virus strains and clinical samples using the QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA was eluted in 60 μl elution buffer and used directly for reverse transcription. First strand cDNA synthesis was performed using Sensiscript® Reverse transcription kit (Qiagen) and random hexameres (Jena Biosciences Gmbl, Jena, Germany) according to the manufacturer's guidelines. Briefly, 5 μL of purified RNA were added to a mixture contained 2 μl of 10× buffer RT, 2 μl (0.5 mM) of dNTPs mix, 2 μl (200 μM) of random hexameres, 1 μl (4 U) RNase inhibitor, 1 μl of sensiscript® Reverse Transcriptase and 7 μl of RNase free water. After incubation at 37 °C for 60 min, the mixture was cooled to 4 °C and stored at −80 °C until use.

### 2.5. Real-time PCR conditions

The SYBR Green I based real-time PCR assay was performed on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using QuantiTect SYBR Green PCR Kit (Qiagen). The assay was carried out in a total volume of 25 μl reaction mixture prepared in triplicates in 96-well optical reaction plates or MicroAmp® optical tubes (Applied Biosystems). The reaction conditions were first optimized by testing variable concentrations of BCoV and internal control QPCR primer sets; $MgCl_2$ concentrations; template volumes and primer annealing

**Table 1**
Viruses used in the study.

| Virus | Abbreviation | Strain | Source | Cell lines used for virus propagation |
|---|---|---|---|---|
| Bovine coronavirus | BCoV | Mebus | Ohio State University, USA | MDBK |
| Bovine rotavirus | BRV | NCDV | Ohio State University, USA | MA104 |
| Bovine rotavirus | BRV | UK | Montreal University, Canada | MA104 |
| Bovine viral diarrhea virus | BVDV | Osloss | South Dakota State University, USA | MDBK |
| Infectious bovine rhinotracheitis virus | IBRV | Cooper | Cairo University, Egypt | MDBK |
| Bovine parinfluenza virus-3 | BPIV-3 | SF | Veterinary Serum and Vaccine Research Institute, Egypt | MDBK |
| Bovine respiratory syncytial virus | BRSV | Egyptian isolate | Central Laboratories for Control of Veterinary Biologics, Egypt | Vero |
| Infectious bronchitis virus | IBV | Mass. H120 | CEVA Sante Animale, Hungary | *Vaccine* |
| Avian Reovirus | ARV | S1133 | Cairo University, Egypt | Vero |
| Human parainfluenza virus-3 | HPIV-3 | Riyadh 11/2008 | King Saud University, Saudi Arabia | Vero |

**Table 2**
Oligonucleotide primers.

| Primers | Role | Sequence (5′–3′) | Length (bp) | Position | PCR product (bp) |
|---|---|---|---|---|---|
| BCoV-SBF (sense) | Real-time QPCR | 5′-TGG ATC AAG ATT AGA GTT GGC-3′ | 21 | 30,371–30,391[a] | 236 |
| BCoV-SBR (antisense) | (BCoV detection and quantification) | 5′-CCT TGT CCA TTC TTC TGA CC-3′ | 20 | 30,588–30,607[a] | |
| BCoV-NF (sense) | Plasmid construction | 5′-GCA TGG ACA CCG CAT TGT TG-3′ | 20 | 29,353–29,372[a] | 1420 |
| BCoV-NR (antisense) | | 5′-CAC CAG GTG CCG ACA TAA GG-3′ | 20 | 30,753–30,772[a] | |
| BGAPDH-F (sense) | Real-time QPCR | 5′-CTG AGT ATG TGG TGG AGT CC-3′ | 20 | 383–402[b] | 178 |
| BGAPDH-R (antisense) | (Internal control) | 5′-GAG GCA TTG CTG ACA ATC TTG-3′ | 21 | 540–560[b] | |

[a] Nucleotide positions based on the complete genome sequence of BCoV strain Mebus, with GenBank accession number U00735.2.
[b] Nucleotide positions based on the Bovine GAPDH mRNA sequence, with GenBank accession number XM_001252511.3.

temperatures. A standard reaction mixture contained 12.5 μl of 2× QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 0.6 μl (0.3 μM) of the following primers (BCoV-SBF, BCoV-SBR, BGAPDH-F and BGAPDH-R), 2 μl of cDNA template and 8.1 μl PCR grade water. The cycling profile involved initial PCR activation step at 95 °C for 15 min, followed by 45 cycles of denaturation at 94 °C for 15 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. The fluorescence was measured at the end of each cycle. Following amplification, a melt curve analysis was performed to verify the specificity of the amplified products by their specific melting temperatures (Tm). Melting curve analysis consisted of a denaturation step at 95 °C for 15 s, decreased to 60 °C for 1 min, and followed by temperature increase to 95 °C at a rate of 1% with continuous reading of fluorescence. Data acquisition and analysis of the results were performed using the 7500 System SDS Software Version 2.0.1 (Applied Biosystems). Each fluorescent reporter signal was measured against the internal reference dye (ROX) signal to normalize for non-PCR related fluorescence fluctuations between wells. The fluorescence threshold limit of the ABI Prism 7500 system was set at 0.02 as recommended by the manufacturer.

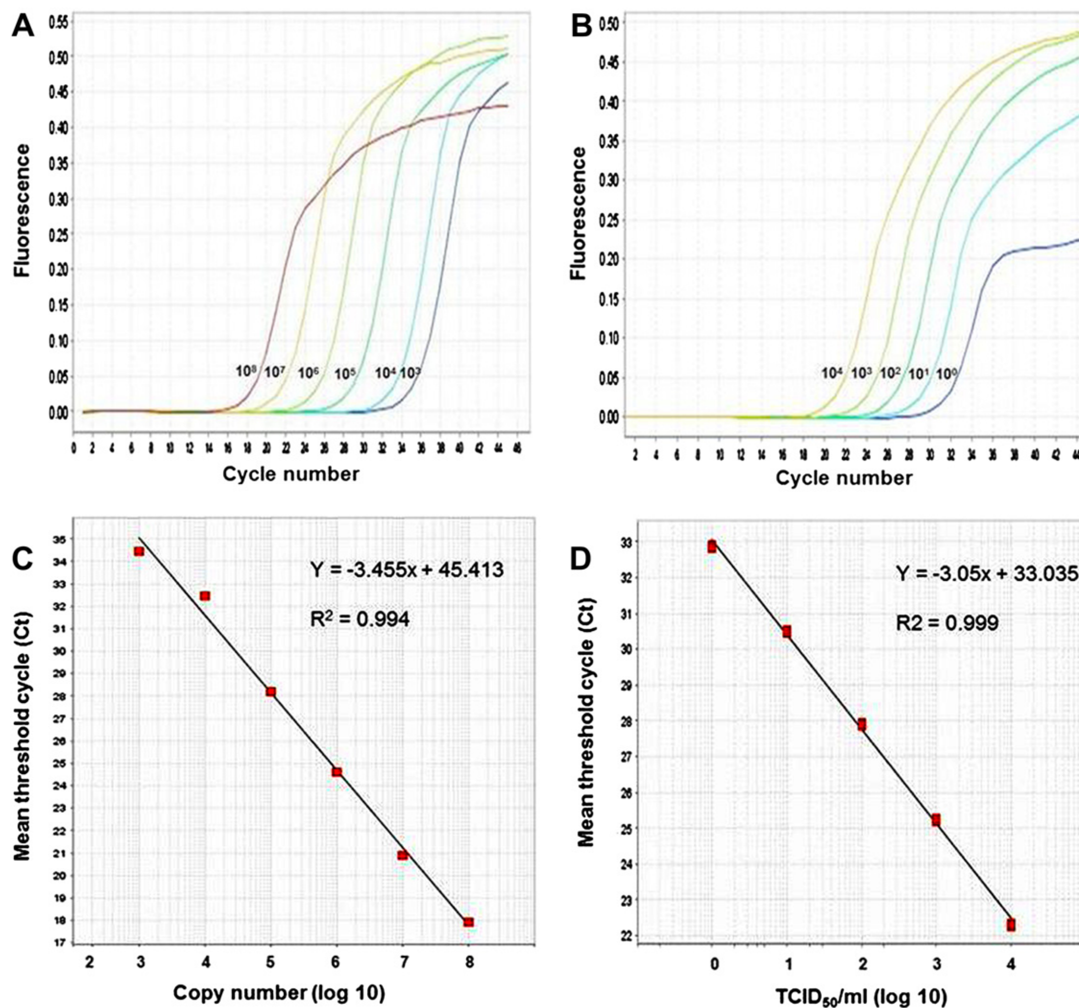


**Fig. 1.** Sensitivity of the SYBR Green I based real-time PCR. Amplification plots (cycle number versus fluorescence) of (A) serially diluted DNA plasmid standards [copies/reaction] and (B) serially diluted cell-culture grown BCoV reference strain [$TCID_{50}$/ml]. Standard curves generated from the mean cycle threshold ($C_T$) values obtained against the (C) diluted DNA plasmid standards (log 10 copy number) and (D) diluted virus strain (log 10 $TCID_{50}$). The coefficient of determination ($R^2$) and the equation of the regression curve ($Y$) were calculated.

**Table 3**
Reproducibility of the BCV real-time RT-PCR assay.

| Assay | Cycle of threshold ($C_T$) for DNA standards (copies/reaction) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Inter-assay variation | | | | | | Intra-assay variation | | | | | |
| | $10^8$ | $10^7$ | $10^6$ | $10^5$ | $10^4$ | $10^3$ | $10^8$ | $10^7$ | $10^6$ | $10^5$ | $10^4$ | $10^3$ |
| 1 | 20.73 | 23.31 | 26.60 | 29.55 | 33.93 | 35.46 | 19.94 | 23.00 | 27.04 | 29.85 | 34.83 | 35.20 |
| 2 | 20.90 | 22.75 | 26.65 | 29.81 | 34.04 | 35.77 | 19.91 | 23.00 | 26.99 | 30.21 | 35.08 | 35.85 |
| 3 | 19.94 | 23.00 | 26.99 | 29.85 | 34.07 | 35.20 | 19.96 | 23.06 | 27.08 | 29.61 | 34.07 | 36.11 |
| Mean $C_T \pm$ S.D[a] | 20.52 ± 0.51 | 23.02 ± 0.28 | 26.75 ± 0.21 | 29.74 ± 0.16 | 34.01 ± 0.07 | 35.48 ± 0.29 | 19.94 ± 0.03 | 23.02 ± 0.03 | 27.04 ± 0.05 | 29.89 ± 0.3 | 34.66 ± 0.53 | 35.72 ± 0.47 |
| CV (%)[b] | 2.50 | 1.22 | 0.79 | 0.55 | 0.22 | 0.80 | 0.13 | 0.15 | 0.17 | 1.01 | 1.52 | 1.31 |

[a] Standard deviation.
[b] Coefficiency of variation.

### 2.6. Generation of quantification standards

A fragment of 1420 bp containing the entire sequence of BCoV N gene was amplified using the primer pair BCoV-NF/BCoV-NR (Table 2) [14]. The RT-PCR product was cloned into pCR®4-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer guidelines. Plasmid DNA was recovered from the transformed *Escherichia. coli* using the QIAprep Spin Miniprep Kit (Qiagen) as directed by the manufacturer and quantified by spectrophotometric analysis. Appropriate clones were verified by restriction endonuclease digestion and DNA sequencing. Plasmid copy numbers were calculated using the formula described by Ke et al. [15],. A master stock containing $5 \times 10^8$ copies/μl was used for preparation of 10-fold dilution series to generate the standard curve and to test sensitivity and reproducibility of the assay.

### 2.7. Assay specificity

The specificity of the developed assay was assessed against viral nucleic acid extracted from a range of animal viruses of clinical and structural relevance to BCoV (Table 1). The analytical specificity was further established by performing melt curve analysis and agarose gel electrophoresis on the final PCR product. Negative controls included fecal and nasal samples collected from uninfected calves, unrelated virus strains like NDV, ARV and HPIV-3 and MilliQ water.

### 2.8. Assay sensitivity

BCoV reference strain was titrated on MDBK cells using the standard methods [16]. Ten-fold serial dilutions of the original virus stock were prepared in 10% BCoV-free nasal/fecal suspension. The viral RNA was extracted from each dilution and was analyzed by the developed two-step real-time PCR assay and simultaneously with conventional PCR to allow better comparison of the analytical sensitivities. The internal BGAPDH control RNA was measured in all dilutions for quality management. Additionally, the sensitivity of the assay was determined by running 10-fold serial dilutions of the plasmid standard in duplicates. The lower limit of detection was defined as the lowest dilution in the 10-fold series that amplified reliably.

### 2.9. Assay precision

To evaluate reproducibility of the assay, the DNA standards ranging from $10^8$ to $10^3$ copies/reaction were tested repeatedly. Three separate dilution series were assayed in a single run for evaluation of the intra-assay variations. On the other hand, the inter-assay variations were measured by testing each dilution in three separate runs. The mean, standard deviation (SD) and coefficient of variation (CV) were calculated separately for each standard DNA dilution based on their $C_T$ values using Microsoft Excel software.

### 2.10. Conventional PCR

For comparative purposes, gel-based PCR was performed on the cDNA preparations of the BCoV 10-fold dilution series (Section 2.7) and the clinical samples. The PCR mixture contained 12.5 μl of 2× Multiplex PCR Master Mix (Qiagen), 0.5 μl (0.2 μM) of each primer (BCoV and internal control qPCR primer sets), 2 μl of the cDNA, and 8.5 μl PCR grade water. Amplification of target sequences was conducted in a thermal cycler (Applied Biosystem) using the following cycling profile: initial activation step at 95 °C for 15 min; 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 57 °C

for 90 s and extension at 72 °C for 90 s; and final extension step at 72 °C for 10 min. PCR products were separated in 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. The specific bands were identified in comparison with 100 bp DNA ladder (ABgene, Epsom, Surrey, UK) and documented using an image analysis system (IMAGO Compact Imaging System, B&L, USA).

## 3. Results

### 3.1. Optimization of the SYBR Green I based real-time PCR assay

In order to determine the optimal conditions for developing a robust SYBR Green I based real-time qPCR assay that detects BCoV and internal control RNA simultaneously, different variables of the reaction were assessed. Such parameters included: magnesium chloride concentration (2.5–4 mM); concentration of the two sets of primers (0.2–1 μM); template volume (1–5 μl); and annealing temperature (55–60 °C). Criteria for selection of the proper measures were the intensity of fluorescence and the number of cycles required to develop a detectable signal ($C_T$ values). Ideal performance of the assay was achieved by the use of Quantitect SYBR Green PCR Master Mix (Qiagen) along with the optimized concentrations of primers and magnesium chloride as mentioned in Materials and methods Section 2.4.

### 3.2. Internal control validation

Specific internal control primers that target unique sequences of the bovine GAPDH mRNA were designed to validate all the reaction steps from RNA extraction to qPCR amplification/detection (Table 2). These primers also confirm that the reaction was performed correctly and that no excess of inhibitors was present in the extracted sample thus excluding false-negatives. Detection of internal control RNA was performed in all experiments, mostly by the use of nasal/fecal suspension prepared from non-infected calves as a matrix. Identification of the internal control signal was achieved by melting curve analysis, where a distinct melting temperature (Tm) of 82.54 ± 0.32 was obtained.

### 3.3. Generation of external standard curve

The entire *N* gene of BCoV was cloned in pCR®4-TOPO plasmid to provide a constant source of standard DNA. The plasmid DNA was accurately quantified by spectrophotometry and a series of 10-fold dilutions starting from a DNA concentration of 25 μg/ml (equivalent to $10^8$ copies per reaction) till a DNA concentration of 25 fg/ml (equivalent to $10^0$ copies per reaction) were prepared. The correlation between the plasmid dilution and the threshold cycle ($C_T$) values in qPCR was analyzed by plotting a standard curve (Fig. 1C). A linear regression relationship was observed with a coefficient of determination ($R^2$) of 0.994 and a slope of −3.455. These values suggest an optimum PCR efficiency and a good correlation between $C_T$ values and template concentrations.

### 3.4. Specificity and sensitivity of BCoV RNA detection

SYBR Green I based real-time PCR is a sequence independent assay that requires conduction of a melt curve analysis on the PCR products. Specific amplification of the BCoV target sequence was identified by the generation of a melt peak at 78.35 °C ± 0.26. Specificity of the reaction was further evaluated for cross reactivity against different viruses that show symptomatic or genetic relationship with BCoV (Table 1). None of these viruses or the negative controls showed specific positive signals rather than the internal control signal consistent with true-negative results. The sensitivity of the assay was evaluated by testing 10-fold serial dilutions of DNA standards ($10^8$ to $10^0$ copies). The results showed that the assay could detect down to $10^3$ copies of DNA standard per reaction. When testing a dilution series of BCoV reference strain in nasal/fecal suspension ($10^5$ $TCID_{50}$/ml, dilutions $10^{-1}$–$10^{-6}$), the obtained detection limit was 1 $TCID_{50}$/ml; equivalent to $1.17 \times 10^{-3}$ $TCID_{50}$/reaction (Fig. 1A,B). In comparison, the conventional RT-PCR was 10 times less sensitive than the real-time assay for both template systems.

### 3.5. Intra- and inter-assay variations

Accuracy and reproducibility of the assay were established basing on the $C_T$ values obtained from testing DNA standards in triplicates; within each run (intra-assay) and in three consecutive runs (inter-assay) (Table 3). The calculated S.D and C.V values for intra-assay variability were low, ranging from 0.03 to 0.53 and from 0.13% to 1.25%, respectively. The inter-assay variability was slightly higher with S.D ranged from 0.07 to 0.51 and C.V ranged from 0.22% to 2.5%.

### 3.6. Linearity and dynamic range

The dynamic range of the developed qPCR assay was evaluated by carrying out 10-fold dilution series of: a) DNA plasmid standard in water; b) BCoV reference strain spiked in nasal/fecal suspension of non-infected calves; and c) BCoV cDNA in water. Against the DNA standard and the BCoV spiked in nasal/fecal suspension, the PCR was able to detect 6 fold differences in the starting template concentration over a range of $10^3$–$10^8$ copies/reaction and $10^5$–$10^0$ $TCID_{50}$/ml, respectively (Fig. 1). However, the BCoV cDNA spiked in water was detected more efficiently to the extent of 8 logs of dilution (Data not shown).

### 3.7. Assay performance on clinical samples

The competence of the developed real-time RT-PCR assay, for accurate detection of BCoV in clinical samples, was evaluated by analysis of 103 swab samples (68 fecal and 35 nasal) in comparison to the gel-based RT-PCR assay (Table 4). The results of both assays were similar for the evaluation of 65 fecal samples (17 positive and 48 negative) and 33 nasal samples (12 positive and 21 negative). However, three fecal and two nasal samples tested negative by gel-based RT-PCR were found to be positive by real-time RT-PCR. None of the samples tested negative by real-time RT-PCR was found positive by the gel-based method. The concentration of BCoV RNA in the analyzed positive samples varied from $2 \times 10^4$ to $1.56 \times 10^8$ copies/μl for the fecal samples, and $3.77 \times 10^2$ to $1.56 \times 10^8$ copies/μl for the nasal samples. It is worthwhile to mention that the samples tested positive in real-time RT-PCR and negative in gel-based assay contained the least virus RNA concentration that

**Table 4**
Comparative analysis of bovine clinical samples using real-time and gel-based RT-PCR assays.

| | Fecal Swabs No. (%) | Nasal Swabs No. (%) | Total No. (%) |
|---|---|---|---|
| Real-time RT-PCR | | | |
| Positive | 20 (29.4) | 14 (40) | 34 (33) |
| Negative | 48 (70.6) | 21 (60) | 69 (67) |
| Gel-based RT-PCR | | | |
| Positive | 17 (25) | 12 (34.3) | 29 (28.2) |
| Negative | 51 (75) | 23 (65.7) | 74 (71.8) |
| Total | 68 | 35 | 103 |

ranged from $2 \times 10^4$ to $3.47 \times 10^4$ for the fecal samples and $3.77 \times 10^2$ to $2.88 \times 10^3$ for the nasal samples.

## 4. Discussion

The use of PCR in the field of virus disease diagnosis has increased to the point that it is now accepted as the new gold standard. Real-time PCR has catalyzed wider acceptance of PCR because it is more rapid, sensitive and reproducible, while the risk of carryover contamination is minimized [17]. In the current study, the potential of real-time PCR was exploited to develop a reliable assay for detection and quantification of BCoV.

The success of any PCR assay is highly dependent on the primer design and its ability to recognize all strains and variants of the target organism. Just as importantly, the designed primers should not cross react with other viruses that may implicate in the same disease form(s). The nucleocapsid (N) gene of BCoV has been shown to be highly conserved due to strong selective structural constraints that limit its revolution [18,19]. Therefore, a wide range of published sequences of BCoV *N* gene were aligned together and against other coronaviruses that belong to the three known antigenic groups. Potential target sequences that were highly conserved in different strains of bovine respiratory and enteric coronaviruses were identified. These sequences, however, share many similarities with some group II coronaviruses that were proposed to originate from BCoV [20–22], a mater that was unavoidable. More than three primer pairs were synthesized and screened for initial evaluation. Of which, a set of primers that amplify a fragment of 236 bp of *N* gene was found to be most sensitive (Table 2).

In addition to the choice of target region, the design of "in-house" real-time PCR protocols requires the optimization of several amplification conditions. The key elements of such optimization include; the magnesium chloride concentration, which allows the polymerase enzyme to function at an optimal level, the concentration of different primers used, which affect the sensitivity and linearity of the assay, and the temperature of primer annealing, which affects the specificity of the reaction [23,24]. The current assay was optimized using the cell-culture grown BCoV, strain Mebus, by varying each of these parameters individually. The optimal amplification conditions were assessed by reporting the fluorescence intensity and the value of threshold cycles ($C_T$).

The use of fecal and nasal swab samples as the most frequent matrices for detection of BCoV in diseased animals constitutes one of the significant problems that may hinder the performance of RT-PCR assays. Considerable existence of the bile salts and the complex polysaccharides in these matrices can either block the enzyme activity or reduce the Magnesium salt availability [25]. Therefore, monitoring PCR inhibitors in the utilized samples is very important to avoid false negative results. One of the most important methods for monitoring PCR inhibitors is the inclusion and/or detection of an internal positive control (IPC) in the test samples [26]. The added advantage of using an IPC is the ability to control and normalize the variable conditions of the assay starting from the efficiency of sample extraction and ending with the real-time amplification/detection. In the process of developing the present assay, a primer set specific for the house keeping gene "Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)" of bovine species was included in the reaction. Specific identification of the IPC product by melt curve analysis revealed a clearly distinguished melt peak of $82.54 \pm 0.32$ °C. Analysis of variance revealed no significant differences in the assay performance between monoplex and duplex reactions in terms of specificity, sensitivity and linearity (data not shown). This implies that co-amplification of the IPC does not affect the detection and quantification of BCoV RNA.

Validation of 'in-house' real-time PCR assays is a crucial aspect that controls the applicability of these assays in clinical and diagnostic purposes. Four cardinal elements of validation were proposed including specificity, sensitivity, reproducibility and linearity [27]. In our assay, all these parameters were considered for evaluation. The specificity of the reaction has been proved by different means including; a) identification of the amplificon as a distinct band of the expected molecular size by agarose gel electrophoresis, b) generation of a consistent melt peak at 78.35 °C ± 0.26 by melt curve analysis, c) lack of cross reactivity with other bovine viruses that may be considered for differential diagnosis with BCoV, d) Lack of cross reactivity with a representative coronavirus (IBV), and e) Absence of regular amplification signals in different types of negative controls (water, nasal/fecal suspensions and some irrelevant human and poultry viruses). Although different types of group II coronaviruses, such as HCV-OC43, PHEV, CRCoV, HECoV, were not included in the specificity evaluation as a result of their unavailability during the assay development, it is highly expected that these viruses can generate positive signals depending on their sequence homology with BCoV target region.

The detection limit of the assay was evaluated by both cDNA copy numbers and $TCID_{50}$. Ten-fold serial dilutions of the DNA standard as well as the virus stock of known titer ($10^5$ $TCID_{50}$/ml) were used to determine the sensitivity and linearity of the assay as well as to generate standard curves applicable for absolute quantification of BCoV in clinical samples. The assay was able to detect 10-fold differences of both targets over 6–8 orders of magnitude. A minimum of $10^3$ cDNA copies of the plasmid standard and $1.17 \times 10^{-3}$ $TCID_{50}$ of the cell-culture grown virus per reaction was recognized (Fig. 1). In addition, the assay showed to be highly reproducible with very limited intra- and inter-assay variations. The calculated CV values for both parameters did not rise over 2.5%. A mean C.V that is lower than 5% is acceptable and indicates that the assay can generate reproducible results [28].

The diagnostic applicability of the developed assay was established by testing this method on clinical samples and comparing its results with the conventional RT-PCR assay. Out of 34 positive samples by real-time RT-PCR (20 fecal and 14 nasal), five samples (three fecal and two nasal) were diagnosed negative by the gel-based assay. As expected, these samples contained the least virus copy number among all positive samples. On the other hand, all the negative samples identified by the real-time assay were also negative by the conventional method. This confirms the higher sensitivity of the developed assay and its reliability for generation of an appropriate epidemiological data. Moreover, the assay proved its ability to detect BCoV over a wide dynamic range (5.5 logs of magnitude for nasal samples and 4 logs for fecal samples). The limited dynamic range of fecal samples as compared to the nasal samples may be explained by the higher background of inhibitory substances in fecal material.

Recently, three reports described the process of establishment of real-time RT-PCR assays for detection of BCoV either solely or in a multiplex set [13,29,30]. However, none of these systems utilized the SYBR Green I chemistry for specific detection and quantification of BCoV. The main advantages of SYBR Green I over other real-time PCR detection formats are; a) it is a low-cost fluorochrome, b) it is a simpler approach especially for primer design and optimization procedures [31], and c) the artifacts commonly observed in specific probes, particularly at amplification cycles beyond the 30th, are minimal and can be ruled out by melt curve analysis [32].

In conclusion, the real-time PCR described here is a sensitive, simple and cost-effective method that can be applied easily for laboratory diagnosis of BCoV infections. The method is convenient for early differential diagnosis of calf diarrhea and bovine

respiratory disease syndromes. In addition, the rapid results obtained by this method are crucial for proper herd management and prevention of disease spread. Moreover, this assay can be considered as a suitable alternative for the regular methods used for studying BCoV pathogenesis and vaccine trials.

## Coauthor contribution

H.M. Amer, H.M. designed the experiments, performed the practical work and revised the manuscript; F.N. Almajhdi developed the concept, interpret the results and wrote the manuscript.

## References


[1] Saif LJ, Heckert RA. Enteric coronavirus. In: Saif LJ, Theil KW, editors. Viral diarrheas of man and animals. Boca Raton: CRC Press; 1990. p. 185–252.
[2] Saif LJ. A review of evidence implicating Bovine coronavirus in the etiology of winter dysentery in cows: an enigma resolved? Cornell Vet 1990;80(4):303–11.
[3] Storz J, Lin X, Purdy CW, Chouljenko V, Kousoulas KG, Enright FM, et al. Coronavirus and *Pasteurella* infections in bovine shipping fever pneumonia and Evans criteria for causation. J Clin Microbiol 2000;38(9):3291–8.
[4] Crouch CF, Oliver S, Hearle DC, Buckley A, Chapman AJ, Francis MJ. Lactogenic immunity following vaccination of cattle with Bovine coronavirus. Vaccine 2001;19(2–3):189–96.
[5] Van Regenmortel MH, Fauquet CM, Bishop DH, Carsten EB, Estes MK, Lemon SM, et al. Virus Taxonomy. Classification and nomenclature of viruses. Seventh report of the International Committee on the Taxonomy of viruses. San Diego: Academic Press; 2000.
[6] Guy JS, Brian DA. Bovine coronavirus genome. J Virol 1979;29(1):293–300.
[7] Lai M, Cavanagh D. The molecular biology of coronaviruses. Adv Virus Res 1997;48:1–100.
[8] Silva RE, O'Reilly KL, Lin X, Stine L, Storz J. Sensitivity comparison for detection of respiratory Bovine coronaviruses in nasal samples from feedlot cattle by ELISA and isolation with the G clone of HRT-18 cells. J Vet Diagn Invest 1999; 11(1):15–9.
[9] Cho KO, Hasoksuz M, Nielsen PR, Chang KO, Lathrop S, Saif LJ. Cross protection studies between respiratory and calf diarrhea and winter dysentery coronavirus strains in calves and RT-PCR and nested PCR for their detection. Arch Virol 2001;146(12):2401–19.
[10] Takiuchi E, Stipp DT, Alfieri AF, Alfieri AA. Improved detection of bovine coronavirus N gene in faeces of calves infected naturally by a semi-nested PCR assay and an internal control. J Virol Methods 2006;131(2):148–54.
[11] Klein D. Quantification using real-time PCR technology: applications and limitations. Trends Mol Med 2002;8(6):257–60.
[12] Watzinger F, Ebner K, Lion T. Detection and monitoring of virus infections by real-time PCR. Mol Aspects Med 2006;27(2–3):254–98.
[13] Escutenaire S, Mohamed N, Isaksson M, Thorén P, Klingeborn B, Belák S, et al. SYBR Green real-time reverse transcription-polymerase chain reaction assay for the genetic detection of coronaviruses. Arch Virol 2007;152(1):41–58.
[14] Amer HM, Hussein HA, El-Sabagh IM, El-Sanousi AA, Saber MS, Shalaby MA. Baculovirus expression of the Bovine coronavirus nucleocapsid protein in *Spodoptera frugiperda* insect cells. Egypt J Virol 2008;5(1):284–303.
[15] Ke GM, Cheng HL, Ke LY, Ji WT, Chulu JL, Liao MH, et al. Development of a quantitative Light Cycler real-time RT-PCR for detection of avian reovirus. J Virol Methods 2006;133:6–13.
[16] Saif LJ, Heckert RA, Miller KL, Tarek MM. Cell culture propagation of Bovine coronavirus. J Tissue Cult Methods 1988;11(3):139–45.
[17] Mackay IM. Real-time PCR in the microbiology laboratory. Clin Microbiol Infect 2004;10:190–212.
[18] Lapps W, Hogue BG, Brian DA. Sequence analysis of the bovine coronavirus nucleocapsid and matrix protein genes. Virology 1978;157:47–57.
[19] Spaan W, Cavanagh D, Horzinek MC. Coronaviruses: structure and genome expression. J Gen Virol 1988;69:2939–52.
[20] Erles K, Toomey C, Brooks H, Brownlie J. Detection of a group 2 coronavirus in dogs with canine infectious respiratory disease. Virology 2003;310:216–23.
[21] Vijgen L, Keyaerts E, Moes E, Thoelen I, Wollants E, Lemey P, et al. Complete genomic sequence of human coronavirus OC43: molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event. J Virol 2005;79:1595–604.
[22] Hasoksuz M, Alekseev K, Vlasova A, Zhang X, Spiro D, Halpin R, et al. Biologic, antigenic, and full-length genomic characterization of a bovine-like coronavirus isolated from a giraffe. J Virol 2007;81:4981–90.
[23] Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, et al. Real-Time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev 2006;19(1):165–256.
[24] Rabenau HF, Kessler HH, Kortenbusch M, Steinhorst A, Raggam RB, Berger A. Verification and validation of diagnostic laboratory tests in clinical virology. J Clin Virol 2007;40:93–8.
[25] Monteiro L, Bonnemaison D, Vekris A, Petry KG, Bonnet J, Vidal R, et al. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. J Clin Microbiol 1997;35(4):995–8.
[26] Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P. Practical considerations in design of internal amplification controls for diagnostic PCR assays. J Clin Microbiol 2004;42:1863–8.
[27] Kubista M, Andrade J, Bengtsson M, Forootan A, Jonáke J, Linda K, et al. The real time polymerase chain reaction. Mol Aspects Med 2006;27:95–125.
[28] Islam A, Harrison B, Cheetam BF, Mahony TJ, Young PL, Walkden-Brown SW. Differential amplification and quantitation of Marek's disease viruses using real-time polymerase chain reaction. J Virol Methods 2004;119:103–13.
[29] Decaro N, Elia G, Campolo M, Desario C, Mari V, Radogna A, et al. Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. J Virol Methods 2008;151:167–71.
[30] Cho YI, Kim WI, Liu S, Kinyon JM, Yoon KJ. Development of a panel of multiplex real-time polymerase chain reaction assays for simultaneous detection of major agents causing calf diarrhea in feces. J Vet Diagn Invest 2010;22(4):509–17.
[31] Aldea C, Alvarez CP, Folgueira L, Delgado R, Otero JR. Rapid detection of herpes simplex virus DNA in genital ulcers by real-time PCR using SYBR Green I dye as the detection signal. J Clin Microbiol 2002;40:1060–2.
[32] Karlsen F, Steen HB, Nesland JM. SYBR Green I DNA staining increases the detection sensitivity of viruses by polymerase chain reaction. J Virol Methods 1995;55:153–6.