
Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods 193 (2013) 337–340

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

# A new approach for diagnosis of bovine coronavirus using a reverse transcription recombinase polymerase amplification assay

H.M. Amer[a,b], A. Abd El Wahed[c,d,*], M.A. Shalaby[b], F.N. Almajhdi[a], F.T. Hufert[c], M. Weidmann[c]

[a] Department of Botany and Microbiology, College of Science, King Saud University, 11451 Riyadh, Saudi Arabia
[b] Department of Virology, Faculty of Veterinary Medicine, Cairo University, 12211 Giza, Egypt
[c] Department of Virology, University Medical Center, 37075 Goettingen, Germany
[d] Department of Virology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt

*Article history:*
Received 3 December 2012
Received in revised form 11 June 2013
Accepted 19 June 2013
Available online 28 June 2013

*Keywords:*
Point-of-care systems
Molecular diagnostic techniques
RPA
Bovine coronavirus

A B S T R A C T

Bovine coronavirus (BCoV) is an economically significant cause of calf scours and winter dysentery of adult cattle, and may induce respiratory tract infections in cattle of all ages. Early diagnosis of BCoV helps to diminish its burden on the dairy and beef industry. Real-time RT-PCR assay for the detection of BCoV has been described, but it is relatively expensive, requires well-equipped laboratories and is not suitable for on-site screening. A novel assay, using reverse transcription recombinase polymerase amplification (RT-RPA), for the detection of BCoV is developed. The BCoV RT-RPA was rapid (10–20 min) and has an analytical sensitivity of 19 molecules. No cross-reactivity with other viruses causing bovine gastrointestinal and/or respiratory infections was observed. The assay performance on clinical samples was validated by testing 16 fecal and 14 nasal swab specimens and compared to real-time RT-PCR. Both assays provided comparable results. The RT-RPA assay was significantly more rapid than the real-time RT-PCR assay. The BCoV RT-RPA constitutes a suitable accurate, sensitive and rapid alternative to the common measures used for BCoV diagnosis. In addition, the use of a portable fluorescence reading device extends its application potential to use in the field and point-of-care diagnosis.



## 1. Introduction

Bovine coronavirus (BCoV) is an important pathogen of livestock causing enteric disease in newly born calves (neonatal calf diarrhea), and adult cattle (winter dysentery) (Clark, 1993). The virus is also implicated in respiratory tract infections of growing calves and shipping fever pneumonia in feedlot cattle (Saif, 2010; Storz et al., 2000). Some BCoV virus strains may also be responsible for simultaneous enteric and respiratory tract infections (Decaro et al., 2008a). Although BCoV infections have a high morbidity that may reach 100%, it rarely causes death. However, the economic losses are substantial in dairy and beef farms due to sudden and dramatic reduction in milk production and loss of body weight (Svensson et al., 2003; Traven et al., 2001).

The current measures used for BCoV diagnosis consist mostly of the detection of viral RNA by conventional and real-time reverse transcription polymerase chain reaction (RT-PCR) (Amer and Almajhdi, 2011; Decaro et al., 2008b; Takiuchi et al., 2006). Samples collected from animals in the field are sent to the laboratory for testing as mobile real-time PCR is rarely available, complex technically and expensive. Therefore, a portable, simple, rapid nucleic acid detection test, and accurate as PCR is needed to detect the virus at the site where infections occur. Recombinase polymerase amplification (RPA) is an isothermal DNA amplification and detection technology (Piepenburg et al., 2006). In contrast to PCR, amplification depends on a specific combination of enzymes and proteins (recombinase, single strand binding protein, and strand displacing DNA polymerase) used at a constant temperature (Euler et al., 2012a,b). Real-time detection of the amplification process is contingent on RPA exo probes and the fluorescence signal is measured in real-time via a simple point-of-care scanner weighing only 1.2 kg including a laptop computer (ESEQuant Tubescanner device, QiagenLake Constance GmbH, Stockach, Germany). In this report, the development of a real-time RT-RPA assay for rapid, simple and portable detection of BCoV in field samples was described.

*Abbreviations:* BCoV, bovine coronavirus; RPA, recombinase polymerase amplification; RT, reverse transcriptase; PCR, polymerase chain reaction; MEM, minimal essential medium; LAMP, loop-mediated isothermal amplification.

* Corresponding author at: Institute of Virology, University Medical Center, Kreuzbergring 57, 37075 Goettingen, Germany. Tel.: +49 551 39 10559; fax: +49 551 39 10552.
*E-mail addresses:* abdelwahed@dpz.eu, abdelwahed@me.com (A. Abd El Wahed).

0166-0934/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jviromet.2013.06.027

## 2. Materials and methods

### 2.1. Cell culture and virus strains

BCoV, Mebus strain (provided by Ohio State University, USA), was grown for 3 days on MDBK cells, and maintained in minimal essential medium (MEM) supplemented with a mixture of penicillin and streptomycin (1%) (Sigma, St. Louis, MO). Other viruses utilized in the study to ascertain the specificity of the assay included bovine rotaviruses (UK strain from Montreal University, Canada, NCDC strain from Ohio State University, USA), bovine viral diarrhea virus (Osolss strain from South Dakota State University, USA), infectious bovine rhinotracheitis (Cooper strain, from Cairo University, Egypt), bovine parainfluenza type 3 virus (SF strain from Veterinary Serum and Vaccine Research Institute, Egypt) and bovine respiratory syncytial virus (Egyptian strain from Central Laboratories for Control of Veterinary Biologics, Egypt).

### 2.2. Clinical samples and RNA preparation

Thirty bovine samples, 16 fecal swabs and 14 nasal swabs, were collected from suspected cases of gastrointestinal and/or respiratory tract infections form farms around Riyadh, Saudi Arabia. BCoV free swabs were obtained from non-infected calves, and tested by real-time RT-PCR, to serve as negative controls. Swabs were placed immediately after collection in 2 ml MEM containing 1000 U penicillin, and 1 mg streptomycin. After transportation on dry ice, samples were mixed by pulse-vortexing for 15 s, and swabs were discarded. Sample suspensions were diluted 1:10 in MEM and centrifuged at 5000 rpm for 15 min at 4 °C. The clarified supernatants were transferred to sterile vials, and aliquots of 140 μl were separated for RNA extraction using QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer instructions.

### 2.3. Generation of RNA standard

The isolated RNA of BCoV was reverse transcribed and amplified using the QIAGEN OneStep RT-PCR kit (Qiagen). The forward primer: 5′-TGGATCAAGATT AGAGTTGGC-3′, and the reverse primer: 5′-CCTTGTCCATTCTTCTGACC-3′ were used to amplify 236 nt of the nucleocapsid (N) gene of BCoV (30371–30607 of Genbank accession number U00735) (Amer and Almajhdi, 2011). The RT-PCR reaction was set as follows: RT step at 50 °C for 30 min, initial activation at 95 °C for 15 min, 30 cycles of 94 °C for 30 s, 58 °C for 60 s, and 72 °C for 60 s, and a final extension step of 72 °C for 10 min. The amplified fragment was ligated into pCR®II using TA-cloning kit dual promoter with One shot® chemically competent *Escherichia coli* (Invitrogen, Darmstadt, Germany). The ligated fragment was confirmed by sequencing (Seqlab, Goettingen, Germany), and RNA was transcribed and quantified using the RiboGreen reagent kit (Invitrogen) as previously described (Weidmann et al., 2003). The standard was tested by a published real-time RT-PCR protocol (Amer and Almajhdi, 2011) using Light Cycler 2.0 and the LightCycler RNA Master SYBR Green I (Roche, Manheim, Germany). The assay performance showed the same sensitivities ($10^3$) as reported in the original publication and the specificity of the real-time RT-PCR was confirmed depending on the melting curve ($T_m$: 78 ± 0.5) (Amer and Almajhdi, 2011).

### 2.4. Real-time RT-RPA amplicon design

The RT-RPA forward primer 5′-GCTATAATGGTGCAATTAG-ATTTGACAGT-3′, and reverse primer 5′-GCTGACGCTGTGG-TTTTGGACTCATATTC-3′ for the detection of BCoV N gene were designed using the following available GenBank sequences (EF193073–EF193074). The RT-RPA amplicon was placed between nt 30451 and 30585 of the GenBank accession number U00735 (length 135 nt). The RPA exo probe was synthesized by TIB MOLBIOL (Berlin, Germany) as following 5′-GGACTCATATTCATCATACCATCTTGTTGT (BHQ1-dT) (THF) (FAM-dT) ATGC ATTCAAATTCTC-Phosphate.

### 2.5. RT-RPA reaction condition

The BCoV RT-RPA was performed in a 50 μl volume using TwistAmp™ exo lyophilized kit (TwistDx, Cambridge, UK). 420 nM RT-RPA primers, 120 nM RT-RPA exo probe, 2 U Transcriptor Reverse Transcriptase (Roche, Mannheim, Germany), 20 U RiboLock RNase inhibitor (Fisher, Schwerte, Germany), 2 μM DTT (Roche), 14 mM Mg acetate, 4× TwistAmp™ rehydration buffer (TwistDx), and 1 μl RNA template were added to the RPA strips containing a dried enzyme pellet. Fluorescence measurements in the FAM channel (Excitation 470 nm, Detection 520 nm) were performed in an ESEQuant tubescanner (Qiagen) at 42 °C for 20 min 8 (Euler et al., 2012a). A combined threshold and signal slope analysis offered by the tubescanner software, was used for signal interpretation that can be confirmed by 2nd derivative analysis.

### 2.6. Determination of sensitivity and specificity

A dilution range from $10^7$ to $10^1$ molecules/μl of the BCoV RNA standard was prepared as described previously (Weidmann et al., 2008). The BCoV RT-RPA was tested using the quantitative RNA standard in 8 replicates, the threshold time was plotted against molecules detected and a semi-log regression was calculated. For exact determination, a probit regression was performed using the Statistica software (StatSoft, Hamburg, Germany). To determine the relationship between real-time RT-PCR CT value and RT-RPA threshold time, a linear regression analysis using Prism software (Graphpad Software Inc., San Diego, California) was performed. The target sequence of GenBank accession number EU401981 was synthesized (Life Technologies/GeneArt AG, Regensburg, Germany) to check a sequence mismatch at position 11 of the upstream primer, which occurs in sequences GenBank accession number FJ938063, EU401980-82, and DQ811784. The assay specificity was evaluated against a panel of viruses considered for differential diagnosis with BCoV. The final assay performance was validated by testing RNA extracts of bovine clinical samples as compared to real-time RT-PCR results.

## 3. Results

### 3.1. BCoV RT-RPA sensitivity and specificity

Using a dilution range of $10^7$ to $10^1$ molecules/μl of the BCoV RNA standard, the sensitivity of real-time RT-PCR was confirmed at $10^3$ molecules detected (Amer and Almajhdi, 2011), while the sensitivity of RT-RPA assay was $10^2$ to $10^1$ molecules (Fig. 1A and B). A probit regression analysis using the results of 8 complete molecular standard runs calculated that in 95% of cases, BCoV RT-RPA could be detected down to 19 RNA molecules (Fig. 1C). A synthesized sequence carrying the mismatch in the upstream primer was also detected and had no influence on the performance of the assay. In recent study, up to nine mismatches within RPA primers and probe did not affect the HIV-1 RPA assay performance (Boyle et al., 2013).

The specificity of BCoV RT-RPA assay was determined by cross detection of other viruses causing bovine gastrointestinal and/or respiratory infections such as bovine rotaviruses, bovine viral diarrhea virus, infectious bovine rhinotracheitis, bovine parainfluenza

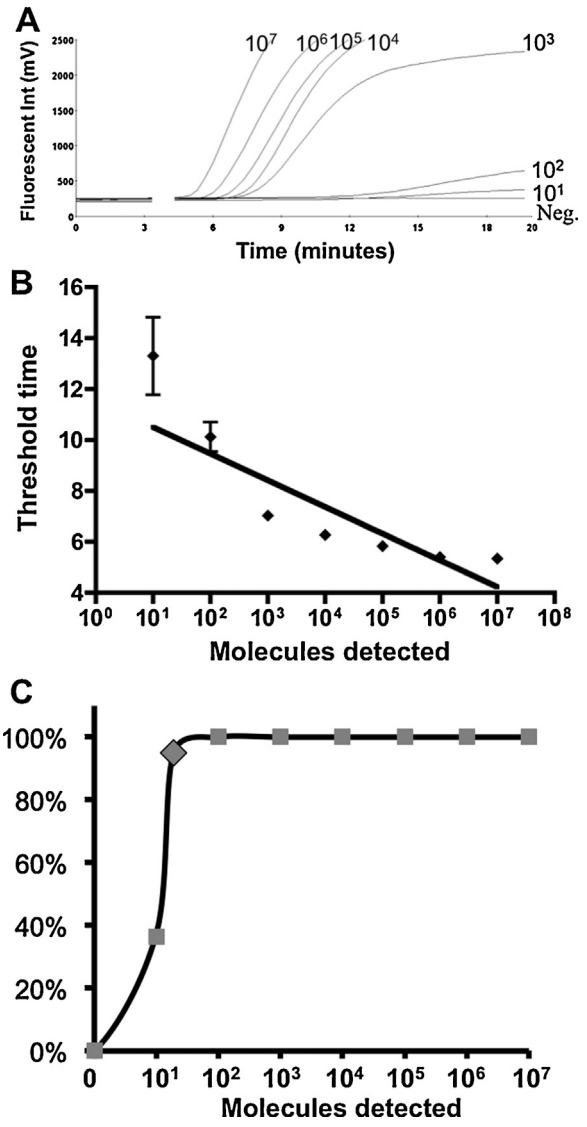


**Fig. 1.** Performance of the real-time BCoV RT-RPA. (A) Fluorescence development over time using a dilution range of $10^7$ to $10^1$ molecules/μl of the BCoV RNA standard (Graph generated by ESEquant tubescanner software). (B) Semi-logarithmic regression of the data collected from 8 BCoV RT-RPA test runs on the RNA standard using Prism Software version 4.0. (C) Probit regression analysis using Statistica software on data of the 8 runs. The limit of detection at 95% probability (19 molecules) is depicted by a rhomboid.

type 3 virus, and bovine respiratory syncytial virus. No cross detections were observed.

### 3.2. Validation of assay performance on clinical samples

Sixteen fecal and 14 nasal swab specimens were collected from cattle showing intestinal and/or respiratory manifestations. The total RNA extracts of these samples were tested with real-time RT-PCR and RT-RPA. Both assays showed the same performance (19 positive and 11 negative cases). The cycle threshold and threshold time values of RT-PCR and RT-RPA respectively well at an $R^2$ value of 0.8 (Fig. 2).

## 4. Discussion

Diagnosis of BCoV as a potential cause of gastroenteritis and respiratory tract infections in cattle is hampered by the implication of a long list of pathogens in these disease syndromes (Snodgrass et al., 1986). The diagnostic difficulty of BCoV is exaggerated by its fastidious growth in cell culture and the relative inaccuracy of most utilized serological assays (Clark, 1993). Molecular approaches including conventional gel-based, nested/semi-nested, and real-time RT-PCR have proved extremely expedient for BCoV detection (Amer and Almajhdi, 2011; Decaro et al., 2008b; Fukuda et al., 2012; Park et al., 2007; Takiuchi et al., 2006; Zhu et al., 2011). However, they require expensive high-precision instruments, considerable expertise, complex optimized experimental setup and specific well-equipped laboratories. Therefore, these assays are not suitable for field use and point-of-care applications.

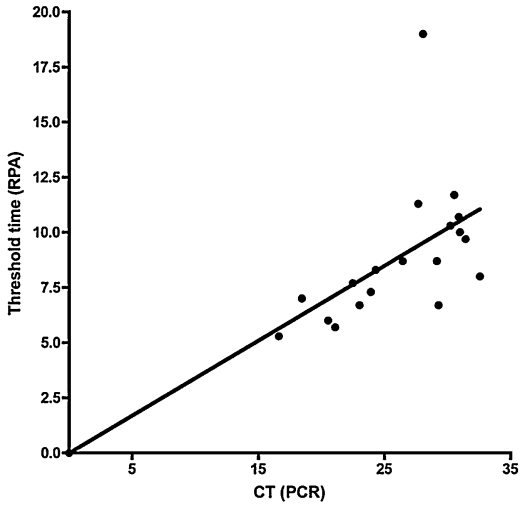


**Fig. 2.** Comparison between performances of RT-RPA and real-time RT-PCR on clinical samples. Thirty RNA extracts of samples collected from suspected cases of BCoV were screened. Linear regression analysis of RT-RPA threshold time (*y* axis) and RT-PCR cycle threshold (CT) values (*x* axis) were determined by Prism software. $R^2$ value was 0.8.

Since the early 1990s, isothermal DNA amplification methods have been developed as a simple, rapid and cost-effective alternative to PCR-based amplification. As they maintain the higher sensitivity and specificity of real-time PCR, the use of isothermal amplification approaches in on-site screening could improve the diagnostic procedures for acute infections. Established isothermal amplification methods include transcription mediated amplification, nucleic acid sequence based amplification, strand displacement methods as loop-mediated isothermal amplification (LAMP), rolling circle amplification methods, helicase dependent amplification, hybridization chain reaction and RPA (Chang et al., 2012; Gill and Ghaemi, 2008; Kim and Easley, 2011). These methods differ in terms of complexity (number of enzymes, primers and/or probes), sensitivity, specificity, speed, contamination risk, portability, and commercial availability. Compared to other isothermal amplification techniques, RPA requires no initial heating for DNA denaturation and the target is amplified exponentially at a constant low temperature (37–42 °C). Together with the availability of assay reagents in a dry pellet form and the use of a battery-charged portable instrument (ESEQuant tubescanner), RPA may be the most applicable approach for field diagnosis of infectious diseases.

In a previous report, a real-time RT-PCR system for detection and quantitation of BCoV using SYBR Green I and melting curve analysis was established (Amer and Almajhdi, 2011). It was designed to target the nucleocapsid (N) gene, which is highly conserved among BCoV strains and of which the subgenomic RNA is the most abundant in coronavirus-infected cells (Spaan et al., 1988). The assay showed a distinct specificity, reproducibility and an analytical sensitivity of $10^3$ copies of the DNA plasmid standard. In this study, an RT-RPA assay for the detection of BCoV was developed targeting the same gene. RNA standard was generated for better evaluation of the

assay performance. While real-time RT-PCR assay maintained the same analytical sensitivity ($10^3$ copies) using the RNA standard, RT-RPA achieved an improved analytical sensitivity (Fig. 1A and B) of 19 molecules detected as determined by probit regression analysis (Fig. 1C). The assay was shown to be reproducible throughout 8 test runs and linear over a range of 7 orders of magnitude, from $10^1$ to $10^7$ RNA copies (Fig. 1A). The diagnostic applicability of the developed RT-RPA assay under field conditions was further validated by testing 30 bovine clinical samples as compared to real-time RT-PCR. The results of both assays were similar for all samples and the values of threshold time (in RT-RPA) and threshold cycles (in real-time RT-PCR) were highly compatible expect for only one sample (Fig. 2).

Recently, an RT-LAMP assay was developed for the detection of BCoV in fecal specimens (Qiao et al., 2012). Although this assay is highly specific and sensitive, it seems to lack several advantages in comparison to the developed RT-RPA assay: (i) ease and flexibility of oligonucleotide design; only two primers and a single probe are required for RT-RPA, while three primer pairs are utilized in LAMP, (ii) significant assay run time advantage; 10–20 min for RT-RPA and 60–90 min for LAMP, (iii) better mobility due to smaller and lighter detection device (iv) additionally the RT-RPA was also evaluated with nasal samples.

In conclusion, a novel RT-RPA assay was developed for rapid and sensitive identification of BCoV in bovine fecal and nasal samples. The applicability of this assay for onsite screening can provide an invaluable tool for prompt diagnosis of disease syndromes caused by BCoV and implementation of the proper strategic and control measures. In addition, the availability of a sensitive, specific and simple diagnostic technique like BCoV RT-RPA will aid in epidemiological surveillance of BCoV and identification of chronic shedders in beef and dairy farms.

## Acknowledgements

The study was funded by the Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia and Department of Virology, University Medical Center, Goettingen, Germany. The authors thank their colleague for fruitful discussions.

## References

Amer, H.M., Almajhdi, F.N., 2011. Development of a SYBR Green I based real-time RT-PCR assay for detection and quantification of bovine coronavirus. Mol. Cell. Probes 25, 101–107.

Boyle, D.S., Lehman, D.A., Lillis, L., Peterson, D., Singhal, M., Armes, N., Parker, M., Piepenburg, O., Overbaugh, J., 2013. Rapid detection of HIV-1 proviral DNA for early infant diagnosis using recombinase polymerase amplification. mBio 4, e00135–e213.

Chang, C.C., Chen, C.C., Wei, S.C., Lu, H.H., Liang, Y.H., Lin, C.W., 2012. Diagnostic devices for isothermal nucleic acid amplification. Sensors (Basel) 12, 8319–8337.

Clark, M.A., 1993. Bovine coronavirus. Br. Vet. J. 149, 51–70.

Decaro, N., Campolo, M., Desario, C., Cirone, F., D'Abramo, M., Lorusso, E., Greco, G., Mari, V., Colaianni, M.L., Elia, G., Martella, V., Buonavoglia, C., 2008a. Respiratory disease associated with bovine coronavirus infection in cattle herds in Southern Italy. J. Vet. Diagn. Invest. 20, 28–32.

Decaro, N., Elia, G., Campolo, M., Desario, C., Mari, V., Radogna, A., Colaianni, M.L., Cirone, F., Tempesta, M., Buonavoglia, C., 2008b. Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. J. Virol. Methods 151, 167–171.

Euler, M., Wang, Y., Nentwich, O., Piepenburg, O., Hufert, F.T., Weidmann, M., 2012a. Recombinase polymerase amplification assay for rapid detection of Rift Valley fever virus. J. Clin. Virol. 54, 308–312.

Euler, M., Wang, Y., Otto, P., Tomaso, H., Escudero, R., Anda, P., Hufert, F.T., Weidmann, M., 2012b. Recombinase polymerase amplification assay for rapid detection of *Francisella tularensis*. J. Clin. Microbiol. 50, 2234–2238.

Fukuda, M., Kuga, K., Miyazaki, A., Suzuki, T., Tasei, K., Aita, T., Mase, M., Sugiyama, M., Tsunemitsu, H., 2012. Development and application of one-step multiplex reverse transcription PCR for simultaneous detection of five diarrheal viruses in adult cattle. Arch. Virol. 157, 1063–1069.

Gill, P., Ghaemi, A., 2008. Nucleic acid isothermal amplification technologies: a review. Nucleosides Nucleotides Nucleic Acids 27, 224–243.

Kim, J., Easley, C.J., 2011. Isothermal DNA amplification in bioanalysis: strategies and applications. Bioanalysis 3, 227–239.

Park, S.J., Lim, G.K., Park, S.I., Kim, H.H., Koh, H.B., Cho, K.O., 2007. Detection and molecular characterization of calf diarrhoea bovine coronaviruses circulating in South Korea during 2004–2005. Zoonoses Public Health 54, 223–230.

Piepenburg, O., Williams, C.H., Stemple, D.L., Armes, N.A., 2006. DNA detection using recombination proteins. PLoS Biol. 4, e204.

Qiao, J., Meng, Q., Cai, X., Chen, C., Zhang, Z., Tian, Z., 2012. Rapid detection of Betacoronavirus 1 from clinical fecal specimens by a novel reverse transcription loop-mediated isothermal amplification assay. J. Vet. Diagn. Invest. 24, 174–177.

Saif, L.J., 2010. Bovine respiratory coronavirus. Vet. Clin. North Am. Food Anim. Pract. 26, 349–364.

Snodgrass, D.R., Terzolo, H.R., Sherwood, D., Campbell, I., Menzies, J.D., Synge, B.A., 1986. Aetiology of diarrhoea in young calves. Vet. Rec. 119, 31–34.

Spaan, W., Cavanagh, D., Horzinek, M.C., 1988. Coronaviruses: structure and genome expression. J. Gen. Virol. 69, 2939–2952.

Storz, J., Lin, X., Purdy, C.W., Chouljenko, V.N., Kousoulas, K.G., Enright, F.M., Gilmore, W.C., Briggs, R.E., Loan, R.W., 2000. Coronavirus and Pasteurella infections in bovine shipping fever pneumonia and Evans' criteria for causation. J. Clin. Microbiol. 38, 3291–3298.

Svensson, C., Lundborg, K., Emanuelson, U., Olsson, S.O., 2003. Morbidity in Swedish dairy calves from birth to 90 days of age and individual calf-level risk factors for infectious diseases. Prev. Vet. Med. 58, 179–197.

Takiuchi, E., Stipp, D.T., Alfieri, A.F., Alfieri, A.A., 2006. 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 131, 148–154.

Traven, M., Naslund, K., Linde, N., Linde, B., Silvan, A., Fossum, C., Hedlund, K.O., Larsson, B., 2001. Experimental reproduction of winter dysentery in lactating cows using BCV – comparison with BCV infection in milk-fed calves. Vet. Microbiol. 81, 127–151.

Weidmann, M., Meyer-Konig, U., Hufert, F.T., 2003. Rapid detection of herpes simplex virus and varicella-zoster virus infections by real-time PCR. J. Clin. Microbiol. 41, 1565–1568.

Weidmann, M., Sanchez-Seco, M.P., Sall, A.A., Ly, P.O., Thiongane, Y., Lo, M.M., Schley, H., Hufert, F.T., 2008. Rapid detection of important human pathogenic Phleboviruses. J. Clin. Virol. 41, 138–142.

Zhu, W., Dong, J., Haga, T., Goto, Y., Sueyoshi, M., 2011. Rapid and sensitive detection of bovine coronavirus and group a bovine rotavirus from fecal samples by using one-step duplex RT-PCR assay. J. Vet. Med. Sci. 73, 531–434.