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Evaluation of a single-tube fluorogenic RT-PCR assay for detection of bovine respiratory syncytial virus in clinical samples

Mikhayil Hakhverdyan[a,*,1], Sara Hägglund[b,1], Lars-Erik Larsen[c], Sándor Belák[a]

[a] *Department of Virology, The National Veterinary Institute, Ulls väg 2B, 751 89 Uppsala, Sweden*
[b] *Department of Ruminant Medicine and Epidemiology, Swedish University of Agricultural Sciences, Uppsala, Sweden*
[c] *Department of Veterinary Diagnostics and Research, Danish Institute for Food and Veterinary Research, Copenhagen, Denmark*

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Abstract

Bovine respiratory syncytial virus (BRSV) causes severe disease in naïve cattle of all ages and is a common pathogen in the respiratory disease complex of calves. Simplified methods for rapid BRSV diagnosis would encourage sampling during outbreaks and would consequently lead to an extended understanding of the virus. In this study, a BRSV fluorogenic reverse transcription PCR (fRT-PCR) assay, based on TaqMan principle, was developed and evaluated on a large number of clinical samples, representing various cases of natural and experimental BRSV infections. By using a single-step closed-tube format, the turn-around time was shortened drastically and results were obtained with minimal risk for cross-contamination. According to comparative analyses, the detection limit of the fRT-PCR was on the same level as that of a nested PCR and the sensitivity relatively higher than that of a conventional PCR, antigen ELISA (Ag-ELISA) and virus isolation (VI). Interspersed negative control samples, samples from healthy animals and eight symptomatically or genetically related viruses were all negative, confirming a high specificity of the assay. Taken together, the data indicated that the fRT-PCR assay can be applied to routine virus detection in clinical specimens and provides a rapid and valuable tool in BRSV research.

Keywords: BRSV; Detection; Fluorogenic RT-PCR; Real-time; TaqMan

1. Introduction

Bovine respiratory syncytial virus (BRSV), a Pneumovirus belonging to the Paramyxoviridae family, is a respiratory pathogen of cattle that causes economical losses to beef and dairy production. The infection consists as a part of a disease complex in calves (Stott et al., 1980), but it also occurs as outbreaks in naïve populations and may be fatal in adults (Elvander, 1996; Inaba et al., 1972). BRSV has enveloped virions; it possesses a negative sense, single-stranded RNA genome and exists as a single serotype. Genomic phylogenetic studies have divided isolates into six subgroups with the highest average percentage of sequence divergence in the glycoprotein (G) coding region of the genome (Valarcher et al., 2000). The fusion (F) protein coding region is less variable and is therefore a more suitable target for the design of diagnostic tests (Eleraky et al., 2003).

Due to problems of detection by classical methods, the importance of BRSV infections in cattle may be underestimated. The period of virus shedding from the point when clinical signs appear is short in infected animals; the virus is labile and therefore difficult to isolate in cell culture (Valarcher et al., 1999; West et al., 1998). BRSV-specific antibodies in clinical specimens may interfere with virus isolation (VI) and also with direct detection methods based on serological techniques (West et al., 1998). Furthermore, the usage of indirect detection by serology (BRSV-specific IgG detection)

* Corresponding author. Tel.: +46 18 674313; fax: +46 18 674669.
E-mail address: mikhayil.hakhverdyan@sva.se (M. Hakhverdyan).
[1] They contributed equally to this work.

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is hampered by the suppressive effect of maternal antibodies on the humoral response to infection (Kimman et al., 1987).

The introduction of the sensitive conventional PCR assays for virus diagnosis has lead to a significant improvement in the detection of BRSV (Larsen et al., 1999; Oberst et al., 1993; Valarcher et al., 1999; Vilcek et al., 1994; West et al., 1998). These methods, however, require complex laboratory manipulations and are therefore time-consuming and expensive (Belák and Thorén, 2001).

The purpose of the present study was to develop a single-step, closed-tube format fluorogenic reverse transcription PCR (fRT-PCR) assay for the detection of BRSV in clinical samples from cattle. The assay had to meet requirements such as high sensitivity, specificity, short detection time and relatively low costs.

2. Materials and methods

2.1. Samples for comparative analyses

Before application of the fRT-PCR assay, lung samples from 28 cattle, submitted for examination to the Danish Institute for Food and Veterinary Research, were tested for BRSV, bovine coronavirus (BCoV) and bovine parainfluenza-3 (PIV-3) virus by antigen ELISAs (Ag-ELISAs) (Uttenthal et al., 1996). Thirty-four lung samples from calves included in a BRSV challenge experiment (Tjornehoj et al., 2003) were tested for BRSV by conventional PCR (Larsen et al., 1999) and/or Ag-ELISA. Nasal swabs from animals included in a second BRSV challenge experiment were also tested by virus isolation (Hägglund et al., in press) ($n = 28$) or nested PCR (Vilcek et al., 1994) ($n = 14$). All samples were analysed in a blind manner.

2.2. Field outbreaks

Clinical samples were obtained from outbreaks of respiratory disease in three Swedish dairy herds (A, B and C). A majority of the animals in these herds showed clinical signs of respiratory disease, including cough and decreased appetite.

In herd A, sera and nasal swabs were obtained from three cows with rectal temperature exceeding 40 °C. In herd B, similar samples were obtained from a calf with cough and normal rectal temperature as well as from a cow with elevated respiratory rate (80 min^{-1}), abnormal sounds on lung auscultation, cough, depression and rectal temperature of 39.6 °C. A swab was also obtained from apical parts of the right lung of a cow that was found dead the previous day. In herd C, nasal swabs were obtained from seven animals with clinical signs ranging between serous nasal discharge alone to cough, rectal temperature up to 41.1 °C, abnormal sounds on lung auscultation and depression. One cow with abdominal, open mouth breathing, subcutaneous emphysema and rectal temperature of 39.6 °C was sampled twice 10 days apart (nasal swabs and sera).

2.3. RNA extraction

RNA was extracted from 250 μl of template (cell culture supernatant, nasal swab transport medium or approximately 400 mg lung tissue homogenated in 400 μl PBS) using TRI REAGENT LS (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. RNA was recovered in 30 μl of sterile, nuclease-free DMPC (dimethylpyrocarbonate) water and either used immediately or stored at −70 °C until further analysis.

2.4. Primers and TaqMan® probe

The oligonucleotide primers and probe against BRSV were designed from the published sequence of the F gene of isolate 394 (GenBank accession no. AF188554), collected from GenBank using NCBI Blast. Alignments of different BRSV isolates/strains were performed using Lasergene software (DNASTAR Inc., version 5, Madison, WI, USA). Primers and probes for the fRT-PCR were selected using Primer Express™ software (version 1.0, Applied Biosystems, Foster City, CA, USA). Compatible primer sets were selected to ensure efficient amplification and detection of BRSV isolates from all subgroups (Valarcher et al., 2000) (for mismatches, see Table 1). The sequences of the primers were (5′–3′): BRSV-F-485F, AAGGGTCAAACATCTGCTTAACTAG (25 bp, forward), BRSV-F-569R, TCTGCCTGWGGGAAAAAAG (19 bp, reverse). The primers were ordered and synthesized at CyberGene AB (Huddinge, Sweden). The TaqMan® probe, termed BRSV-F-TaqMan-546, had the following sequence (5′–3′): AGAGCCTGCATTRTCACAATACCACCCA (34 bp, complementary). The probe was labelled with 6-carboxyfluorescein (FAM) at the 5′ end and with nonfluorescent Black Hole Quencher Dye at the 3′ end (BHQ, Biosearch Technologies, Novato, CA, USA). The reverse primer and the probe were designed so that they could tolerate one mismatch. The predicted product size of the BRSV amplicon was 85 bp.

2.5. Procedure and efficiency

The fRT-PCR was carried out on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and optimised by titration series of primers, probe and Mn^{2+}. The assay was carried out in a total volume of 25 μl using a 96-well optical reaction plate or MicroAmp® optical tubes (Applied Biosystems). Each 25 μl reaction mixture contained 2 μl of extracted RNA in DMPC water, 0.2 μM of each BRSV primer and probe, 0.5 mM 4X dNTPs, 2.5 mM $Mn(AOc)_2$, 2.5 U/μl of rTth DNA polymerase (Applied Biosystems), 5× EZ buffer and 1 mg/ml of BSA. The final volume was adjusted with DMPC water. The thermodynamic profile used was as follows: 42 °C for 5 min (data collected) and 60 °C for 30 min (RT step), and then 95 °C for 30 s (initial denaturation) followed by 40 cycles of amplifi-

Table 1
Nucleotide mismatches at primer and probe sites of 29 BRSV, one ORSV and one HRSV F-gene sequences (GenBank)

GenBank accession number	Forward primer (BRSV-485F) 25 bp A A G G G T C A A A C A T C T G C T T A A C T A G	Probe (BRSV-F-TaqMan-546)[a] 28bp A G A G C C T G C A T T R T C A C A A T A C C A C C C A	Reverse primer (BRSV-569R)[a] 19bp T C T G C C T G W G G G A A A A A A G
14 BRSV sequences	. . . . .	. . . . .	. . . . .
AF1885771	. . . . .	. . . . . A	. . . . .
AF1885772	. . . . .	. . . . . A	. . . . .
AF1885773	. . . . .	. . . . . A	. . . . .
AF1885774	. . . . .	. . . . . A	. . . . .
AF1885775	. . . . .	. . . . . A	. . . . .
AF1885776	. . . . .	. . . . . A	. . . . .
D00953	. . . . .	. . . . . A	. . . . . A . . . . .
M82816	. . . . .	. . . . . A	. . . . . T . . . . .
AF188567	. . . . .	. . . . .	. . . . . G .
AF188572	. . . . .	. . . . .	. . . . . A . . . . .
AF188573	. . . . .	. . . . .	. . . . . A . . . . .
AF188574	. . . . .	. . . . .	. . . . . A . . . . .
AF188575	. . . . .	. . . . .	. . . . . A . . . . .
AF188576	. . . . .	. . . . .	. . . . . T . . . . .
AF295543	. . . . .	. . . . .	. . . . T . . . . .
AF334398 (ORSV)[b]	. G . . A . . . . . T . . T	. . . A . . . . . G	. . . . . T . . . . .
NC_001781 (HRSV)[c]	. . . . A . . . . . T . . T . . T . . . . . A . .	T . . T . . . . . A . . . . . T . . T	. . A . . . . . A . . G . . G .

[a] Complementary sequence
[b] Ovine respiratory syncytial virus
[c] Human respiratory syncytial virus

cation: 94 °C for 30 s, 50 °C for 30 s (data collected), 72 °C for 45 s.

A 10-fold dilution series of a BRSV positive RNA template (three dilutions, five replicates of each dilution) were used for generation of a standard curve and calculation of the reaction efficiency (E), using formulae $E = 10^{(-1/a)} - 1$, where a is the slope value of the standard curve.

2.6. Acceptance criteria and evaluation of reproducibility

A cycle threshold (Ct) value (the number of cycles for the fluorescence to reach a threshold) of 40.0 was chosen as a negative cut-off for the fRT-PCR assay. As described previously (Reid et al., 2002), any sample that did not generate a sigmoid amplification curve was retested. Duplicate analyses of 62 lung samples assessed the intra-assay reproducibility.

2.7. Detection limit and analytical specificity

The detection limit of the fRT-PCR was determined by testing nine steps of a 10-fold dilution series of a BRSV strain (no. 9402022) (Viuff et al., 1996) with a concentration of $10^{4.5}$ $TCID_{50}$/ml. Total RNA was extracted from each dilution and was analysed by fRT-PCR (two replicates of each dilution, from 10 to 10^9) and in parallel by nested PCR (Vilcek et al., 1994). The experiment was repeated with both assays. The analytical specificity of the assay was evaluated by testing nine BRSV strains originating from the Netherlands (37 NL, 1974), Hungary (908/8 H, 1975), Sweden (271 SW, 4556 SW, 187 SW, 504 SW, 1992–1995) and Denmark (195 DK, 1988; 4899 DK, 1993; 2020 DK, 1994), nasal swabs from 14 healthy calves and 8 virus species, genetically or symptomatically related to BRSV. The viruses included bovine coronavirus ($n = 3$), parainfluenza-3 virus ($n = 7$), bovine viral diarrhoea virus, type 1 (BVDV-1; $n = 2$), border disease virus (BDV; $n = 1$), bovine herpesvirus, type 1 (BHV; $n = 1$), bovine reovirus, types 1 and 3 ($n = 2$), bovine adenovirus, types 1 and 6 (BAV; $n = 2$) and human RSV, types A and B (HRSV; $n = 3$).

3. Results

3.1. Efficiency, amplicon size and reproducibility

As calculated from the slope of the standard curve ($a = -3.434$; Fig. 1) and the formulae described above, the efficiency of fRT-PCR was 0.96 or 96%. This means that, on average, the number of DNA copies increased by 96% in every cycle. During control visualization of fRT-PCR products from six positive and one negative sample in agarose gel, the size of the bands from all positive samples agreed to the predicted size of 85 bp and the negative sample did not yield a visible band. Moreover, results of the 62 samples that were tested in duplicate were in complete accordance. Obtained Ct values for positive samples ($n = 21$) differed with 0.03–1.8 cycles (mean variation 0.4).

3.2. Comparative analyses, sensitivity and specificity studies

When testing dilution series of a BRSV strain ($10^{4.5}$ $TCID_{50}$/ml, dilutions 10–10^9), amplification curves with increasing Ct-values were achieved for each dilution from 10 to 10^4 (Fig. 2). The obtained detection limit was accordingly 3.16 $TCID_{50}$/ml. Results of the same RNA templates tested by BRSV nested PCR were identical with those obtained by fRT-PCR (Fig. 3). The experiment was repeated and results were the same (data not shown).

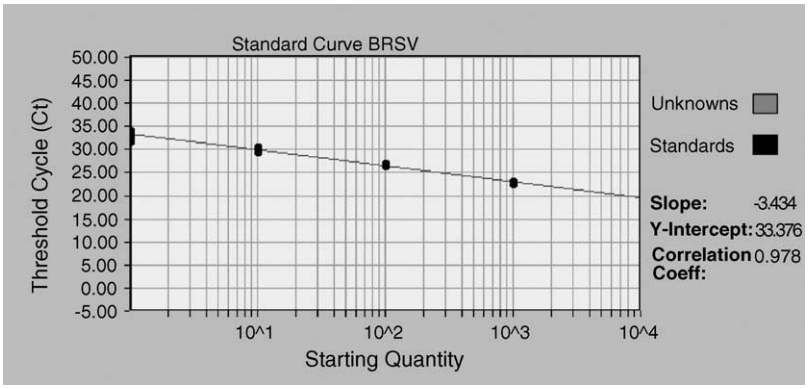

Fig. 1. Standard curve for calculation of BRSV-fRT PCR efficiency. The curve is generated by analyses of a 10-fold dilution series of an RNA template (dilutions 1:1 to 10^3, five replicates of each dilution).

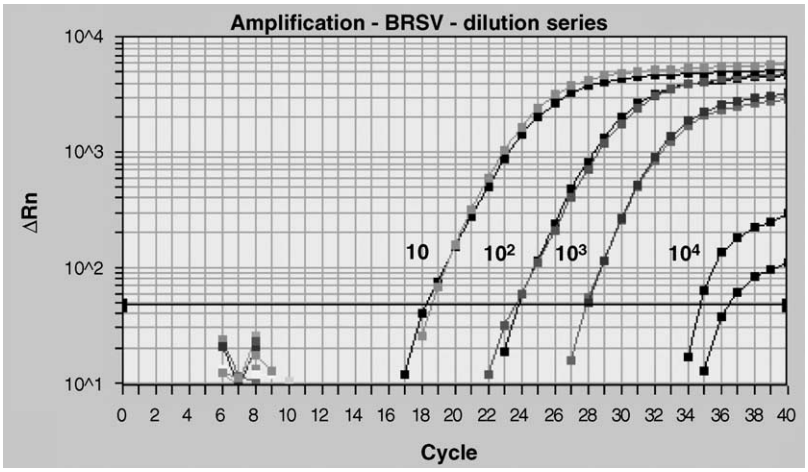

Fig. 2. Determination of detection limit for BRSV fRT-PCR. Nine steps of a 10-fold dilution series of a BRSV strain, with an initial titre of $10^{4.5}$ $TCID_{50}$/ml, were tested (dilutions 10–10^9, two replicates of each dilution). The detection limit is 10^4 times diluted sample.

The results of the fRT-PCR, Ag-ELISA, conventional PCR, nested PCR and VI were in accord with 137 of 147 analyses on 101 samples. The results for the rest 10 analyses were as follows: the fRT-PCR detected 4 more positive samples than Ag-ELISA, 3 more than conventional PCR and 2 more than VI. Only nested PCR detected 1 more positive sample than fRT-PCR (Table 2). The relative diagnostic sensitivity and specificity percentages are shown in Table 3.

Investigations on the analytical specificity comprised two tests. In the first test, nine BRSV strains (see above), human RSV (types A and B), as well as positive and negative controls, were included. All BRSV strains and the positive controls were amplified successfully, but not human RSV or the negative controls (data not shown). In the second test, symptomatically or genetically related virus species (see above), as well as three different BRSV positive and two negative controls, were used. Only the positive controls yielded positive results (Fig. 4).

3.3. Field outbreaks

All nasal swabs from dairy cows with acute respiratory infections in herd A were positive for BRSV by the fRT-PCR assay and identical results were obtained by the nested PCR.

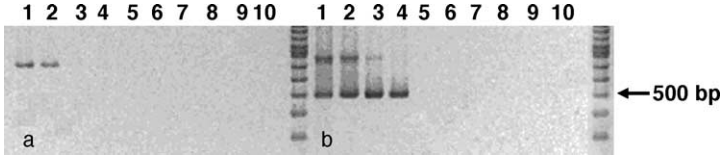

Fig. 3. BRSV nested PCR results from analyses on the same RNA templates as described in Fig. 2. The products of PCR 1 (a) and PCR 2 (b) are shown by gel electrophoresis. Predicted product size is 711 bp for PCR 1 and 481 bp for PCR 2. The detection limit is 10^4 times diluted sample (number 4 on gel b). Numbers 1–9 correspond to the dilutions from 10 to 10^9; number 10 is negative control.

Table 2
Comparison of diagnostic results on field samples and on samples from animals included in BRSV challenge experiments

Origin[a]	Sample id	No.[b]	Virus isolation	Nested PCR	fRT-PCR	Ag-ELISA	Conv.[c] PCR
Lung (field), $n = 28$	1–12	12	na	na	+	+	na
	13–27	15	na	na	–	–	na
	28	1	na	na	+	–	na
Lung (exp), $n = 34$	29–34	6	na	na	+	+	+
	35–57	23	na	na	–	–	–
	58–59	2	na	na	+	+	–
	60	1	na	na	+	–	–
	61–62	2	na	na	+	–	na
NS (field), $n = 11$	63–70	8	na	+	+	na	na
	71–72	2	na	–	–	na	na
	73	1	na	+	–	na	na
NS (exp), $n = 14$	74–82	9	+	+	+	na	na
	83–86	4	–	–	–	na	na
	87	1	–	+	+	na	na
NS (exp), $n = 14$	88–95	8	+	na	+	na	na
	96–100	5	–	na	–	na	na
	101	1	–	na	+	na	na
Total[d]			28	25	101	62	32

na, not analysed.
[a] Origin and type of samples; field samples or samples from experimentally infected animals. Nasal swabs (Virocult®, Medical Wire and Equipment Co. Ltd., England).
[b] Number of samples.
[c] Conventional PCR.
[d] Total number of analyses per method.

Table 3
Relative sensitivity and specificity of BRSV fRT-PCR

Assay		No. of analyses ($n = 147$)	Type of samples (origin)	Results[c]				Relative sensitivity (%)[d]	Relative specificity (%)[d]
A	B			A+ B+	A+ B–	A– B+	A– B–		
fRT-PCR	Ag-ELISA	62	Lung (field), $n = 28$ Lung (exp[a]), $n = 34$	20	4	0	38	100	90.5
fRT-PCR	Conventional PCR	32	Lung (exp[a])	6	3	0	23	100	88.5
fRT-PCR	Nested PCR	25	NS[b] (field), $n = 11$ NS[b] (exp[a]), $n = 14$	18	0	1	6	94.7	100
fRT-PCR	Virus isolation	28	NS[b] (exp[a]), $n = 28$	17	2	0	9	100	81.8

[a] Experimentally infected animals.
[b] Nasal swabs (Virocult®, Medical Wire and Equipment Co. Ltd., England).
[c] A+ B+; assays A and B positive, A+ B–; assays A positive and B negative, A– B+; assays A negative and B positive, A– B–; assays A and B negative.
[d] Obtained for assay A when assay B is taken as the reference.

In herd B, nasal swabs from both a calf with mild respiratory signs and a cow with pneumonia were tested positive by fRT-PCR. In addition, BRSV was detected in a swab from the lung of a dead animal. In herd C, nasal swabs from four animals out of seven were tested positive by fRT-PCR. The positive samples were derived from two young animals with mild cough and serous nasal discharge, and from two dairy cows with severe pneumonia. Three samples, which were negative in fRT-PCR, were obtained from cows with serous nasal discharge as the only observed clinical sign of disease. One of these three samples was positive in nested PCR. Nasal swabs from a cow with severe pneumonia, obtained 10 days apart, were positive in both PCR assays. Acute sera from animals in all three herds were negative for BRSV-specific IgG antibodies and a seroconversion to BRSV was detected in paired sera in all cases.

4. Discussion

The conventional laboratory diagnosis of BRSV is based on virus isolation, antigen/antibody ELISA, immunohistochemistry and PCR. At present, the most sensitive diagnostic tool for direct diagnosis is the nested PCR because it includes two rounds of amplification with two discrete primer sets, which increase drastically the limit of detection (Belák and Thorén, 2001). Although the nested PCR is undoubtedly effective, the practical application is rather complicated. It

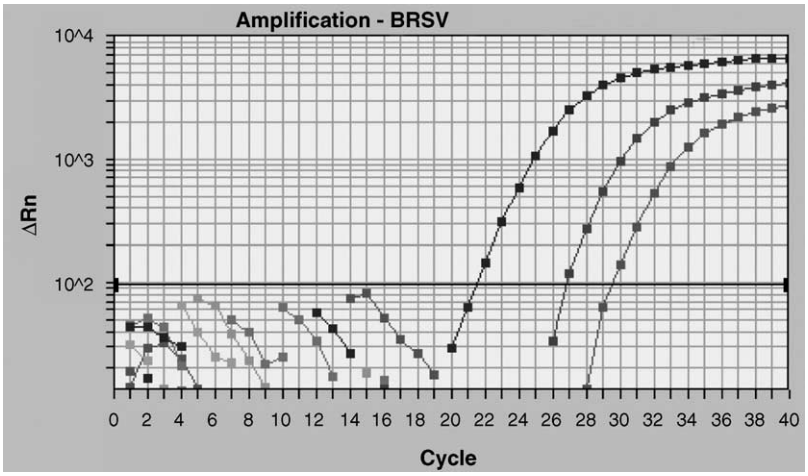

Fig. 4. BRSV fRT-PCR specificity test with symptomatically or genetically related viruses (BCoV, PIV-3, BVDV-1, BDV, BHV-1 1 and 3, bovine reovirus, BAV-1 and 6) and three positive controls. Only the positive controls were detected (Ct values 21.5, 26.9, 29.5).

takes five laborious steps to achieve the final results: reverse transcription, two rounds of amplification, gel electrophoresis and photography. The second round of amplification not only delays the results, but also increases the risks of cross-contamination. Moreover, gel electrophoresis and photography include handling of ethidium bromide, which with its carcinogenic effects hazards the health of laboratory personnel.

Considering the needs for a sensitive and specific, as well as safer and more rapid method for the diagnosis of BRSV infections, we have developed a single-step, closed-tube fRT-PCR assay for the detection of this virus in clinical samples. To our knowledge, this is the first report on the development and application of an fRT-PCR system for the direct diagnosis of respiratory diseases caused by BRSV. Although Werling et al. (2002) published recently a BRSV TaqMan PCR assay, their method was developed for research purposes, in order to measure viral mRNA levels in the context of cytokine responses. Our intention was to develop a simple and high throughput fRT-PCR assay and to investigate its diagnostic applicability by testing clinical samples from experimentally and naturally infected animals. Compared to nested PCR, the single-tube fRT-PCR assay has important advantages. By including rTth DNA polymerase, which acts both as a thermoactive reverse transcriptase and a thermostable DNA polymerase, a separate RT step was avoided and the reactions require less than 3 h to complete (from RT reaction to results), compared to 7–8 h for RT and nested PCR. Unlike other protocols, neither the addition of a second enzyme nor an alteration in buffer composition is required between the reverse transcription and the PCR.

The fRT-PCR allows a considerably increased sample throughput, which is a very important requirement in diagnostic laboratories. During simultaneous examination of up to 96 samples, the results of duplicate tests were in complete agreement. Negative controls included in each run gave no amplification signals, suggesting that cross-contamination was avoided. With future development of standardized protocols for different respiratory pathogens, the sample capacity of this assay will allow specimens to be screened for several targets in a single run.

As another important requirement for a diagnostic assay, the BRSV fRT-PCR has provided a high analytical specificity. The assay gave negative results when testing samples obtained from healthy animals or heterologous viruses related to BRSV symptomatically or genetically. The lack of amplification with human RSV types A and B, together with positive results of all BRSV strains, showed that the assay was highly specific.

According to the current data, the detection limit of the fRT-PCR was on the same level as that of a nested PCR. Only one lung sample amongst 25 tested was positive in nested PCR and negative in fRT-PCR. In the rest cases, PCR results were in good agreement, whereas the fRT-PCR showed higher relative sensitivity compared to that of Ag-ELISA, conventional PCR and virus isolation on a limited number of samples. The positive fRT-PCR results on lung and nasal secretions from experimental animals, with negative results in other assays (Table 2), were probably true positive, since all these animals had been inoculated with BRSV.

Divergent results between fRT-PCR and VI might be explained by interfering BRSV-specific antibodies in clinical specimens. Antibodies may block virus and impede isolation attempts (West et al., 1998). Indeed, the animal from which samples with divergent results were obtained possessed high BRSV-specific antibody titres in nasal secretions (Hägglund et al., in press). These samples also generated the highest Ct-values of all positive samples detected by fRT-PCR (data not shown), suggesting that antibodies interfered with VI when viral concentrations in samples were low.

A benefit of fluorogenic PCR systems for virus research is the possibility for quantification of the nucleic acid molecules in samples. In this study, the increasing Ct-values, in agreement with the BRSV dilutions, implied that a relative quan-

tification was functioning already in the developed BRSV fRT-PCR system. For a numeric quantitation of viral copies, however, simultaneous detection of a house-keeping gene in RNA extractions would be required (Gueudin et al., 2003).

The practical use of the fRT-PCR was tested on a carefully selected collection of clinical samples, representing various cases of respiratory infections in cattle. In the field data presented above, animals with acute respiratory signs including high fever or dramatically elevated respiratory rates were tested positive, inferring that this type of clinical cases represent a suitable target group for sampling. It should be noted that calves, but not adults, shed detectable levels of BRSV even with mild clinical signs of disease. BRSV was detected in samples obtained 10 days apart from one animal, indicating that the period of possible detection by fRT-PCR is reasonably long during natural BRSV infections in naïve cattle. Nevertheless, to be assured of a reliable herd diagnosis, nasal swabs should be obtained from more than one animal during an outbreak.

The results suggest that the fRT-PCR assay described above allows a rapid, sensitive and specific detection of BRSV from clinical samples. The detection of targets without opening the reaction vessel and combination of reverse transcription with PCR in a single closed-tube format greatly reduces both the risk for cross-contamination of specimens and the detection time. The sensitivity of the fRT-PCR assay was comparable to that of nested PCR and seemed higher than that of VI or Ag-ELISA. Taking in account that nested PCR is laborious and demands several practical steps to reach the final results, fRT-PCR with its single-step performance is a more suitable tool for detection of BRSV. Another advantage is that multiple results can be obtained with minimal effort. In summary, the results described above indicate that the method is sensitive, simple and can be applied to routine diagnosis.

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