

## Real-time PCR for quantitation of bovine viral diarrhea virus RNA using SYBR Green I fluorimetry

E. KOSINOVA, I. PSIKAL, B. ROBESOVA, K. KOVARCIK

Veterinary Research Institute, Brno, Czech Republic

**ABSTRACT:** Quantitative real-time RT-PCR (qRT-PCR) assay was developed for the detection and quantification of bovine viral diarrhea virus (BVDV) in clinical samples from persistently infected cattle. qRT-PCR was optimized to quantify the number of BVD virus copies using Light Cycler<sup>®</sup> detection system and intercalation fluorogenic dye SYBR Green I. A universal set of primers was selected from a highly conserved 5' untranslated region (5'UTR) to detect BVDV type I and II simultaneously. Quantification of BVDV cDNA was accomplished using a calibration curve generated from 10-fold serial dilutions of standard plasmid DNA in the range 1–10<sup>8</sup> copies/μl. Analysis of 290 bp amplicons enabled monitoring of the viral RNA/BVDV level in a total of five BVDV strains (BVD-NADL, A03/3004, DB03/2943, KA04/3124, KV05/3412) and sixteen bulk milk samples, and in bovine sera of persistent carriers originating from Czech farms, as well as in a batch of calf serum for cell culture. Melting temperatures of amplicons (T<sub>m</sub>) of BVDV strains of the same genotype group I as the NADL reference strain showed variability of the thermal points, however significant differences were observed in T<sub>m</sub> values between the representatives of genotype group I and II. Low concentrations of BVD virus in bulk milk samples were also qualitatively identified by conventional RT-PCR. Highly reproducible data were obtained as the coefficients of variation of threshold cycles values in intra-assay and inter-assay were less than 0.85% and 2.76%, respectively. The results give enough evidence of suitability of qRT-PCR assay for quantitative analysis of BVDV in clinical samples.

**Keywords:** bovine viral diarrhea virus; RNA; real-time RT-PCR; SYBR Green I

Bovine viral diarrhea virus (BVDV) is a member of the *Pestivirus* genus, and causes various clinical syndromes in cattle including diarrhea, mucosal disease, reproduction disfunctions and/or hemorrhagic syndrome (Baker, 1995). Acute and persistent BVDV infections of pregnant cows are often accompanied by BVD virus transmission into the fetuses in which the infections may result in abortions, teratogenic changes or a delivery of persistently infected, immunotolerant calves, depending on the stage of gestation (Brownlie, 1991). The immunotolerant cattle can shed the virus via secretion and excretion into the environment for a long time, thus being a major source of BVDV infections in herds (Baker, 1987). Sensitive diagnostic methods for rapid identification and elimination of persist-

ent carriers in the herds are therefore of significant importance (Lindberg and Alenius, 1999). Common detection of the virus in clinical samples is carried out using standard techniques of isolation in cell cultures or identification of viral antigens by immunoperoxidase and immunofluorescence tests or by demonstration of BVDV antigens in serum by ELISA. Some of these methods are laborious and less sensitive, other are expensive due to high-cost examinations of individual animals. Therefore molecular analytical methods have been commonly used for the detection of BVD virus in different clinical samples using RT-PCR followed by visualization on agarose gels. These methods are more sensitive than common ELISA assays (Horner et al., 1995), but are prone to contamination. The newly

Supported by the Ministry of Agriculture of the Czech Republic (Grants No. 1B44021 and MZE 0002716201).

developed RT-PCR assays are based on combined performing of RNA reverse transcription and monitoring of cDNA amplification using light signal from fluorophores in one tube. Real-time detection of nucleic acid amplification has been used not only for detection, but also for quantification of the target nucleic acid and for the assessment of variability in nucleotide sequences in the amplified region (Heid et al., 1996). Quantitative real-time RT-PCR (qRT-PCR) methods, which have also been developed for application in clinical diagnosis, are predominantly based on differently labeled specific probes using hydrolysis (TaqMan). The released fluorescent light signal is used for quantification and genotyping of BVDV templates (Bhudevi and Weinstock, 2001; Mahlum et al., 2002; Heath et al., 2003; Letellier and Kerkhofs, 2003); in contrast SYBR Green I dye binds non-specifically to double-stranded DNA by intercalation or minor groove binding (Lekanne Deprez et al., 2002; Zipper et al., 2004). Specific identification may be achieved by melting curve analysis that can be used for detecting target nucleic acids characterized by sequence variability.

Our goal was to implement and verify a simple method using a real-time qRT-PCR assay with the intercalating dye SYBR Green I for genetic identification of RNA templates and quantification of cDNA of both cyto- and non-cytopathogenic BVD viruses in infected cell cultures and clinical samples of persistently infected cattle.

## MATERIAL AND METHODS

### Viruses and clinical samples

Five BVDV strains (BVD-NADL, A03/3004, DB03/2943, KA04/3124, KV05/3412), propagated in MDBK cells, were used for optimization of RT-PCR and qRT-PCR assays (Table 1). These samples (except for KV05/3412) represented genotype I according to nucleotide sequences from 5' untranslated region (UTR) of viral genome. Genotype II was represented by KV05/3412 strain. The whole region of 5' UTR sequences was determined by sequencing in all the strains (unpublished). RNA templates from a heterogeneous virus (porcine reproductive and respiratory syndrome virus, PRRSV) or total RNA from non-infected MDBK cells were used in amplifications as negative controls; dH<sub>2</sub>O was used as a contamination control of reaction mixtures.

In the second part of the study, a total of sixteen field samples (cell fractions of bulk milk, bovine blood sera, batch of calf serum) isolated from persistently infected cattle from Czech herds were tested by standard RT-PCR specific for BVDV detection (Table 1). Persistently infected cattle was determined based on two repeated sampling of clinical materials carried out within three weeks, which was repeatedly serologically negative by ELISA, and virologically positive by conventional RT-PCR. Negative samples of bulk milk (BVM-176/05, BVM-203/05) and serum (BS-1/06, BS-39/06) were included in the study as the specificity controls of the method qRT-PCR. The samples were obtained from a BVDV-free herd, and their negativity was repeatedly determined by serological and virological methods. All samples were analysed in triplicate by the optimised qRT-PCR assay.

### Extraction of total RNA

Total RNA from BVDV-infected cell culture suspensions and somatic cells of bulk milk samples was isolated using RNeasy Mini Kit, and that of bovine serum samples (150 µl) was isolated by QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Cell fractions of bulk milk samples were obtained by the procedure described by Drew et al. (1999). The isolated RNA was eluted in 50 µl in DEPC-treated H<sub>2</sub>O and stored at –70°C.

### Conventional RT-PCR assay

Conventional RT-PCR was performed using *C. therm.* Polymerase One-Step RT-PCR System (Roche Diagnostic) according to the manufacturer's instructions. Primers (primer 324: 5'-ATGCCCTTAGTAGGACTAGCA-3' and 326: 5'-TCAACTCCATGTGCCATGTAC-3') used for RT-PCR were designed by Vilcek et al. (1994). Final concentration of each primer was 0.3 µM in reaction. Reverse transcription was performed with 5 µl of the total RNA at 60°C for 30 min, followed by a short incubation at 94°C for 2 min to inactivate the reverse transcriptase and to denature cDNA. After the RT, PCR consisted of 35 cycles of denaturation at 94°C for 30 s, annealing of primers at 55°C for 1 min and extension at 70°C for 1 min. The final step included extension at 70°C for 7 min.

Table 1. Set of BVDV strains, bulk milk samples and bovine sera analyzed by conventional RT-PCR and qRT-PCR methods. Values of copies cDNA/BVDV in  $\mu\text{l}$  and  $T_m$  obtained from qRT-PCR assays

Sample	Material	RT-PCR	cDNA/BVDV (copies/ $\mu\text{l}$ )	Mean $T_m \pm \text{S.D.}$ ( $^{\circ}\text{C}$ )
BVD-NADL*	virus	+	$1.14 \times 10^6$	$86.34 \pm 0.29$
MA03/3004*	virus	+	$4.22 \times 10^4$	$87.14 \pm 0.22$
DB03/2943*	virus	+	$3.89 \times 10^4$	$87.76 \pm 0.26$
KA04/3124*	virus	+	$1.27 \times 10^3$	$86.57 \pm 0.22$
KV05/3412**	virus	+	$1.55 \times 10^5$	$86.96 \pm 0.30$
BVM-11/05	bulk milk	+	98.30	$85.09 \pm 0.21$
BVM-37/05	bulk milk	+	12.17	$85.08 \pm 0.25$
BVM-44/05	bulk milk	+	30.12	$85.73 \pm 0.29$
BVM-57/05	bulk milk	+	15.72	$85.52 \pm 0.30$
BVM-62/05	bulk milk	+	23.04	$85.41 \pm 0.22$
BVM-74/05	bulk milk	+	28.17	$85.68 \pm 0.25$
BVM-131/05	bulk milk	+	33.21	$85.94 \pm 0.20$
BVM-145/05	bulk milk	+	115.30	$85.77 \pm 0.27$
BVM-156/05	bulk milk	+	47.23	$85.97 \pm 0.26$
BVM-159/05	bulk milk	+	67.09	$85.55 \pm 0.23$
BVM-176/05	bulk milk	–	N.D.	N.D.
BVM-203/05	bulk milk	–	N.D.	N.D.
BS-1/06	serum	–	N.D.	$76.30 \pm 0.22$
BS-5/06	serum	+	421.25	$87.33 \pm 0.29$
BS-6/06	serum	+	460.31	$87.17 \pm 0.20$
BS-11/06	serum	+	423.15	$87.15 \pm 0.22$
BS-14/06	serum	+	411.64	$87.10 \pm 0.29$
BS-21/06	serum	+	473.54	$87.42 \pm 0.22$
BS-39/06	serum	–	N.D.	N.D.
PTS-30/05	batch of calf serum	+	23.27	$85.12 \pm 0.27$

$T_m$  = melting temperature, S.D. = standard deviation, N.D. = not detected

\*genotype I, \*\*genotype II

and cooling to  $4^{\circ}\text{C}$ . PCR products were detected in 2% agarose gel in TBE buffer and visualized by ethidium bromide (0.5  $\mu\text{g}/\text{ml}$ ) staining.

### Preparation of standard curves

Standard curves were generated from 10-fold serial dilutions of the recombinant plasmid DNA (pDNA/K15) and purified viral RNA (BVD-NADL strain). Construction of the recombinant plas-

mid DNA was made up using RT-PCR product of 290 bp amplified from 5' UTR region of BVD virus (BVD-NADL strain, genotype I) which was cloned to a plasmid vector pCR II-TOPO (Invitrogen). The recombinant plasmid pDNA/K15 was propagated and purified from competent cells *Escherichia coli* TOP10F'. Plasmid DNA was purified using QIAprep® Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions and its concentration was determined by measuring absorbance at 260 nm. The standard curve was subsequently

used for quantification of BVDV cDNA in field samples.

### Real-time RT-PCR assay

Quantitative RT-PCR was carried out using the SuperScript<sup>TM</sup> III Platinum<sup>®</sup> Two-Step qRT-PCR Kit with SYBR<sup>®</sup> Green (Invitrogen) according to the manufacturer's instructions. RNA templates (1 µl) were reverse transcribed at 25°C for 10 min followed by incubation at 42°C for 50 min using SuperScript III Reverse Transcriptase, random hexamers (2.5 ng/µl), oligo dT (2.5 µM) and MgCl<sub>2</sub> (5mM) (all from Invitrogen). The reverse transcription reaction was stopped by heating the samples to 85°C for 5 min. Next, 2 U of *E. coli* RNase H (Invitrogen) was added, and the reaction mixture was incubated at 37°C for an additional 20 min.

Real-time PCR was performed in Master Mix in a total volume of 20 µl. In each reaction, the Master Mix contained Platinum SYBR Green qPCR SuperMix-UDX (1×), Bovine Serum Albumin (1 µg/µl), 3.5 mM MgCl<sub>2</sub>, and primers UTR\_DL1F and UTR\_DL4R (each at a final concentration 0.2 µM). The primers UTR\_DL1F (5'-GCC ATG CCC TTA GTA GGA CTA GC-3') and UTR\_DL4R (5'-CAA CTC CAT GTG CCA TGT ACA GC-3') were designed from 5'UTR BVDV genome which is identical for both genotype I and II strains (Kim and Dubovi, 2003). The PCR products were amplified from 2 µl cDNA. The PCR was initiated with denaturing step at 95°C for 120 s followed by 45 cycles of denaturation at 94°C for 5 s, annealing at 57°C for 10 s, and elongation at 72°C for 10 s. After the final cycle, analysis of melting temperature (*T<sub>m</sub>*) was carried out in all the amplified samples including the controls by continuous recording of fluorescence at

gradual increase of temperature (0.2°C/s) over the range 55–95°C. The reactions were carried out on a capillary system of LightCycler<sup>®</sup> (Roche Diagnostic) and data analysed using the LightCycler Software version 3.5.

## RESULTS

### Conventional RT-PCR assay

Using the standard protocol of RT-PCR allowed us to amplify the target fragment from the viral genome and obtain PCR products of 288 bp for BVD viruses of both genotypes I and II. All twenty five samples (except for negative samples mentioned in Material and Methods) which were processed by conventional RT-PCR yielded positive amplification (Table 1). Negative amplification was recorded in samples of RNA templates from porcine reproductive and respiratory virus, non-infected MDBK cells and dH<sub>2</sub>O, which were used in amplification as negative controls (Figure 1).

### Standard curves

The standard curve for viral load quantification was generated by amplification of the diluted recombinant pDNA/K15 in the range of 1–10<sup>8</sup> copies per 1 µl, and the number of plasmid DNA copies expressed as log<sub>10</sub> was plotted against the measured threshold cycle (*C<sub>T</sub>*) values. The standard curve determined the relative number of BVDV cDNA copies in a linear range from 1.2 to 1.1×10<sup>8</sup> per 1 µl. Regression coefficients (*R*<sup>2</sup>) between the logarithmic number of copies of plasmid DNA and *C<sub>T</sub>* were repeatedly higher than 0.990, and the

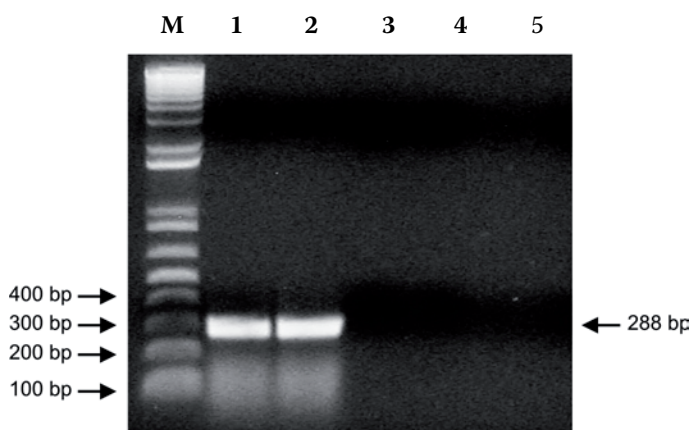


Figure 1. Electrophoresis of PCR products of BVDV 5'UTR obtained by conventional RT-PCR on 2% agarose gel. The length of PCR product was 288 bp. MM = 1Kb Plus DNA Ladder (Invitrogen), 1 = BVD-NADL strain (genotype I), 2 = KV05/3412 strain (genotype II), 3 = PRRSV, 4 = non-infected MDBK cells, 5 = dH<sub>2</sub>O

Table 2. Variability of crossing point values at quantitation of a standard plasmid cDNA/BVDV in 10-fold serial dilutions

Concentration of plasmid cDNA/BVDV (copies/ $\mu$ l)	Intra-assay variability CP (mean $\pm$ S.D.)/CV (%)	Inter-assay variability CP (mean $\pm$ S.D.)/CV (%)
$7.8 \times 10^5$	$15.78 \pm 0.131/0.83$	$15.94 \pm 0.300/1.88$
$6.2 \times 10^4$	$19.72 \pm 0.167/0.85$	$19.54 \pm 0.342/1.74$
$5.6 \times 10^3$	$23.45 \pm 0.005/0.21$	$23.0 \pm 0.617/2.65$
$6.0 \times 10^2$	$26.94 \pm 0.206/0.76$	$27.14 \pm 0.754/2.76$

CP = crossing point, CV = coefficient of variation, S.D. = standard deviation

amplification efficiency (E) ranged according to the slope of a straight line from  $-3.431$  to  $-3.588$  (Figure 2). Reproducibility of the assay was verified in four times repeated analyses with diluted plasmid cDNA/BVDV for determination of variation coefficients (CV) within one assay (intra-assay) and between different assays (inter-assay). The highest CV value was 0.85% and 2.76% in intra-assay and inter-assay, respectively (Table 2).

Quantification of RNA/BVDV was tested in relation to four times repeated analyses of RNA isolated from 10-fold dilutions of BVD virus (BVD-NADL strain) propagated in MDBK cell culture up to the infection dose  $10^{5.3}$  TKID<sub>50</sub> per ml. Number of cycles corresponding with crossing point was directly proportional to the infection dose logarithm and a standardized number of BVDV cDNA copies were originally added into the reaction. Standard RNA/BVDV curve was of linear character ranging between  $8.2$  and  $7.7 \times 10^5$  copies in  $1 \mu$ l (Figure 2). Under these conditions, the sensitivity of RNA/BVDV detection in one real-time RT-PCR reaction was equivalent to 10 copies of cDNA/BVDV in  $1 \mu$ l and infection dose  $\sim 0.02$  TKID<sub>50</sub>.

### qRT-PCR assay

All twenty five samples, which are summarized in Table 1 and were tested by conventional RT-PCR analysis, were also examined by qRT-PCR. Positive amplification by qRT-PCR was observed in all amplified samples, the result of which was a specific PCR product of 290 bp and a fluorescent signal with sigmoidal shape. Negative fluorescent signal was demonstrated qualitatively in samples of negative control of amplification and the negative samples (BVM-176/05, BVM-203/05, BS-1/06, BS-39/06) that eliminated the possibility of false positive results (Table 1).

The results obtained by qRT-PCR and conventional RT-PCR showed high agreement in the demonstration of genetic structures of BVD virus in twenty five samples under testing. Low concentrations of BVD virus in bulk milk samples determined by the number of copies standardized based on the standard plasmid cDNA/BVDV, were qualitatively identified even by the conventional RT-PCR. The results showed in Table 1 indicate that qRT-PCR assay monitors reliably the levels of viral RNA/BVDV not only in bulk milk samples and persistently in-

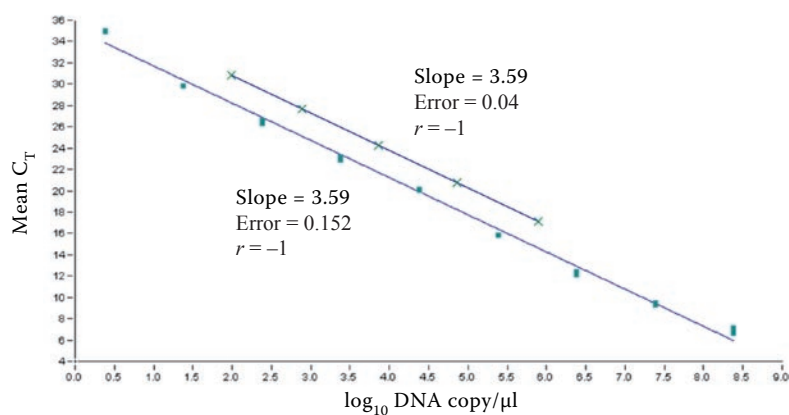


Figure 2. Standard curve generated from 10-fold dilutions of a standard plasmid cDNA BVDV/NADL (squares) and RNA BVD/NADL (crosses) amplified by real-time qRT-PCR. The mean  $C_T$  values were plotted against the dilutions



fectected bovine sera, but also in a batch of calf serum for cell cultures (sample PTS-30/05).

After the amplification, all the samples were subjected to melting temperature analysis. Melting temperatures of amplicons ( $T_m$ ) were presented by plotting the values of negative derivation of the fluorescence signal against temperature. Ten fold repeated analysis of melting curves of the amplified genome segment of BVD-NADL reference strain yielded one expected dissociation peak of  $T_m$  86.34°C, indicating specific amplification (Figure 3a).  $T_m$  values of other BVDV positive sam-

ples, which were classified phylogenetically into the same genotype group I as BVD-NADL strain, showed minor variability of melting temperature points. The highest difference (by 1.42°C) was found in melting temperature of BVDV strain DB03/2943 compared to  $T_m$  of BVD-NADL (Figure 3b). Rather surprisingly for KV05/3412 strain, a representative of genotype II, the dissociation peak  $T_m$  86.96°C of the specific products was obtained. Comparison of  $T_m$  peaks in representatives of both genotypes (I and II) did not reveal any significant differences in melting temperatures (Table 1).

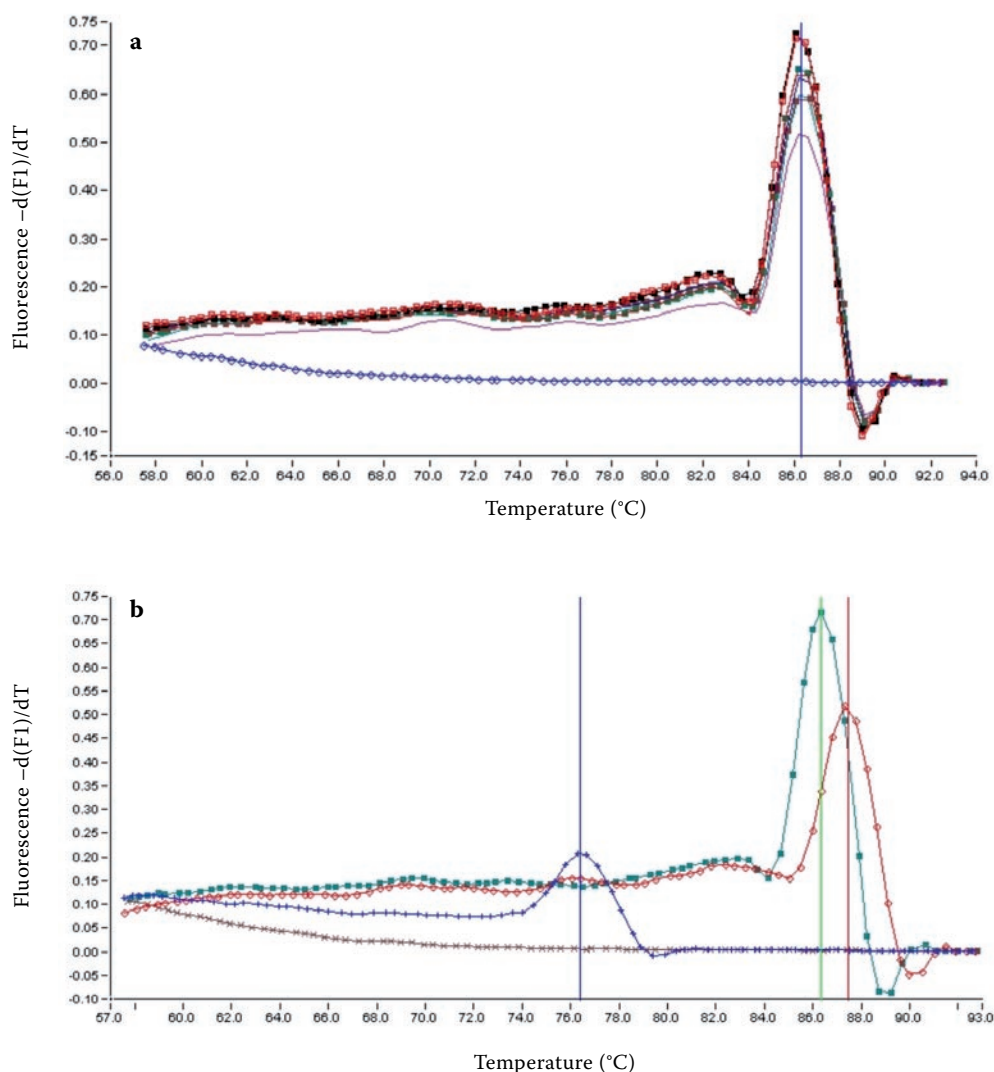


Figure 3. Dissociation plots of BVDV samples after amplification in the SYBR green-based qRT-PCR assay. Temperature on the  $x$  axis is plotted against the negative derivation of the measured fluorescence signal on the  $y$  axis after finished amplification. (a) Repeated analysis of melting curves of BVD-NADL reference strain (squares) yielded one expected dissociation peak of  $T_m$  = 86.34°C. Negative control dH<sub>2</sub>O was marked by dots. (b) Perpendicular with plotted melting points of BVDV specific PCR products of BVD-NADL strain ( $T_m$  = 86.34°C) – squares, DB03/2943 strain ( $T_m$  = 87.76°C) – dots, nonspecific products of sample BS-1/06 ( $T_m$  = 76.30°C) – lines, and negative control (dH<sub>2</sub>O) – crosses

Variability of the  $T_m$  dissociation points in all qRT-PCR analyzed specific PCR products amplified from RNA templates of BVDV strains, somatic cells of bulk milk samples and field bovine sera was about 2.68°C, which can be explained by genetic changes (point mutation, insertion, deletion etc.) in viral RNA of the genome. The values of dissociation peaks of  $T_m$  in BVDV strains ranged from 86.34°C (BVD-NADL) to 87.76°C (DB03/2943) and the number of cDNA/BVDV copies were in orders of magnitude  $10^3$ – $10^6$  per  $\mu$ l. No significant difference was found between  $T_m$  values of positively amplified samples of field bovine sera and BVDV strains, however the differences of  $T_m$  values between bulk milk samples and BVDV strains differed significantly (Table 1).

For quantification of BVDV cDNA the standard curve in field samples was used. The number of copies of cDNA BVDV in  $\mu$ l in Table 1 showed the highest values in a group of BVDV strains (orders of magnitude  $10^3$ – $10^6$ ) unlike of bulk milk samples (orders of magnitude  $10^1$ – $10^2$ ) and positive sera (order of magnitude  $10^2$ ).

## DISCUSSION

Removal of persistently infected calves from the herd is an essential component of programmes for the control of BVDV infection. Conventional RT-PCR has been described for a rapid detection (Hyndman et al., 1998; Renshaw et al., 2000) and genotyping (Gilbert et al., 1999; Vilcek et al., 1999) of BVD virus. The developed real-time PCR assay has some advantages compared to the conventional PCR; it is an important diagnostic tool yielding reliable and reproducible results, does not require post-PCR analysis (gel electrophoresis, hybridization), and the risk of cross contamination is limited (Mackay, 2004). The disadvantage of the real-time PCR assay compared to the conventional PCR might be the cost of the instrument. Two common approaches to the detection of the target amplicon exist, i.e. through specific and nonspecific fluorescence chemicals. Both the techniques have similar sensitivity (Wittwer et al., 1997; Bustin and Nolan, 2004). Detection methods based on SYBR Green I are reliable tools for identification of the target nucleic acid which is characterized by sequence variability, unlike of analyses using probes as TaqMan which require high complementation in the probe binding site. Consequently false negative results in virus

detection can be caused by high sequence variability in the probe binding regions. Other advantages of real-time qRT-PCR assays based on SYBR Green I include simple detection of false or nonspecific amplifications, the ability to detect non-described variants, and reduced time of analysis (Papin et al., 2004; Richards et al., 2004; Young et al., 2006).

Two step real-time qRT-PCR with SYBR Green I labeling and melting curve analysis of 290 bp amplicon has been used in this study for a reliable identification and monitoring of BVDV nucleic acid levels from field samples of bovine blood sera, cell fractions of bulk milk and even batch of calf serum for cell culture. Further, BVDV strains obtained from persistently infected cattle which correspond to genotype I according the sequences from 5'UTR of viral genome, and KV05/3412 strain representing genotype II, were included into the analysis. Repeated monitoring of  $T_m$  values in the representatives of genotype group I (BVD-NADL strain) and II (KV05/3412 strain) did not reveal any significant differences. Such a result can be explained by low variability of the dissociation values  $T_m$  of the amplified fragment of 5'UTR of BVDV viral genome. This genomic region composed of about 400 nucleotides belongs to the most conserved part of the pestivirus genome, and has been used for genetic typing of BVDV strains into two genotype groups (Pellerin et al., 1994; Ridpath et al., 1994; Vilcek et al., 2001). Except for the strain KV05/3412 we did not succeed to obtain more genotype II isolates for our study which would help to clarify the negative result in genotype I and II differentiation. Comparison of 5'UTR sequences of the references strain BVD-NADL and the strain KV05/3412 showed a sequence homology of only 75% between the type I and II viruses, which is in accordance with the findings of Donis (1995).

The set of universal primers designed for a conventional RT-PCR (Vilcek et al., 1994) and qRT-PCR (Kim and Dubovi, 2003) used for detection and genotyping of BVD virus (type I and II) from a highly conserved region 5'UTR differed only in four nucleotides, respectively. Amplicon size belongs to substantial factors which allow differentiation of BVD virus. Shorter fragments have higher specificity of strain differentiation (Varga and James, 2006). Distinguishing between genotype 1 and 2 representatives would make the amplification of a shorter fragment more efficient and specific compared to 290 bp size fragment used in our study. Young et al. (2006) detected a product

of 156 bp from 5'UTR using qRT-PCR assay with SYBR Green I from the samples of whole blood collected from cattle with acute BVDV infection. A reliable detection and BVDV classification into genotype I and II has repeatedly been observed only in the studies using qRT-PCR method and specific probes (Heath et al., 2003; Letellier and Kerkhofs, 2003; Baxi et al., 2006; Hoffmann et al., 2006).

A complete translocation of SYBR Green I dye in amplicons was monitored in a series of melting temperature measurements in the same sample (BVD-NADL strain), which verified the sensitivity, reproducibility and reliability of the method. This can be proved by the values of variation coefficients of intra- and inter-assays and by linear course of both parallel standard curves BVDV/RNA and BVDV/cDNA. High specificity of the reaction was confirmed by detection of nonspecific amplifications, which differed markedly from the specific ones based on significantly lower melting points. This synthesis of nonspecific PCR product can be initiated by the formation of the structure primer-dimer, which is a disadvantage while using an intercalation dye in qRT-PCR. Another assessment parameter of the test is sensitivity of the reaction which allows detection of the number of RNA molecules copies of highly diluted virus in clinical materials. Saliki et al. (1997) investigated the amount of BVDV virus secretion in animal sera which ranged from  $10^3$  to  $10^5$  cell culture infective doses per ml. This level of viral secretion in sera of persistent cattle corresponded with our results. The mentioned values allow to predict the dynamics of occurrence of the target viral RNA in clinical materials of persistently infected carriers whose prevalence in herds ranges from 0.5% to 2.0% (Roeder and Harkness, 1986; Houe, 1999).

In conclusion we can state that the real-time qRT-PCR assay is a highly sensitive method for quantification and determination of BVDV variability level. Quantification of BVD virus copies is not commonly used as a diagnostic parameter but virus concentration can be useful for quantification in different clinical samples and for risk assessment of BVDV transmission among cattle herds.

## REFERENCES

- Baker J.C. (1987): Bovine viral diarrhea virus: a review. *Journal of the American Veterinary Medical Association*, 190, 1449–1458.
- Baker J.C. (1995): The clinical manifestations of bovine viral diarrhea infection. *The Veterinary Clinics of North America. Food Animal Practice*, 11, 425–445.
- Baxi M., McRae D., Baxi S., Greiser-Wilke I., Vilcek S., Amoako K., Deregt D. (2006): A one-step multiplex real-time RT-PCR for detection and typing of bovine viral diarrhea viruses. *Veterinary Microbiology*, 116, 37–44.
- Bhudevi B., Weinstock D. (2001): Fluorogenic RT-PCR assay (TaqMan) for detection and classification of bovine viral diarrhea virus. *Veterinary Microbiology*, 83, 1–10.
- Brownlie J. (1991): The pathways for bovine virus diarrhoea virus biotypes in the pathogenesis of disease. *Archives of Virology*, 3 (Suppl.), 79–96.
- Bustin S.A., Nolan T. (2004): Pitfalls of quantitative real-time reverse transcription polymerase chain reaction. *Journal of Biomolecular Techniques*, 15, 155–166.
- Donis R.O. (1995): Molecular biology of bovine viral diarrhea virus and its interactions with the host. *The Veterinary Clinics of North America. Food Animal Practice*, 11, 393–423.
- Drew T.W., Yapp F., Paton D.J. (1999): The detection of bovine viral diarrhoea virus in bulk milk samples by the use of a single-tube RT-PCR. *Veterinary Microbiology*, 64, 145–154.
- Gilbert S.A., Burton K.M., Prins S.E., Deregt D. (1999): Typing of bovine viral diarrhea viruses directly from blood of persistently infected cattle by multiplex PCR. *Journal of Clinical Microbiology*, 37, 2020–2023.
- Heath G.S., King D.P., Turner J.L., Wakeley P.R., Banks M. (2003): Use of an internal standard in a TaqMan nested reverse transcription-polymerase chain reaction for the detection of bovine viral diarrhoea virus. *Veterinary Microbiology*, 96, 357–66.
- Heid C.A., Stevens J., Livak K.J., Williams P.M. (1996): Real time quantitative PCR. *Genome Research*, 6, 986–994.
- Hoffmann B., Depner K., Schirrmeier H., Beer M. (2006): A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. *Journal of Virological Methods*, 136, 200–209.
- Horner G.W., Tham K.M., Orr D., Ralston J., Rowe S., Houghton T. (1995): Comparison of an antigen capture enzyme-linked assay with reverse transcription – polymerase chain reaction and cell culture immunoperoxidase tests for the diagnosis of ruminant pestivirus infections. *Veterinary Microbiology*, 43, 75–84.
- Houe H. (1999): Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. *Veterinary Microbiology*, 64, 89–107.



- Hyndman L., Vilcek S., Conner J., Nettleton P.F. (1998): A novel nested reverse transcription PCR detects bovine viral diarrhoea virus in fluids from aborted bovine fetuses. *Journal of Virological Methods*, 71, 69–76.
- Kim S.G., Dubovi E.J. (2003): A novel simple one-step single-tube RT-duplex PCR method with an internal control for detection of bovine viral diarrhoea virus in bulk milk, blood, and follicular fluid samples. *Biologicals*, 31, 103–106.
- Lekanne Deprez R.H., Fijnvandraat A.C., Ruijter J.M., Moorman A.F. (2002): Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. *Analytical Biochemistry*, 307, 63–69.
- Letellier C., Kerkhofs P. (2003): Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhoea virus. *Journal of Virological Methods*, 114, 21–27.
- Lindberg A.L., Alenius S. (1999): Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. *Veterinary Microbiology*, 64, 197–222.
- Mackay I.M. (2004): Real-time PCR in the microbiology laboratory. *Clinical Microbiology and Infection*, 10, 190–212.
- Mahlum C.E., Haugerud S., Shivers J.L., Rossow K.D., Goyal S.M., Collins J.E., Faaborg K.S. (2002): Detection of bovine viral diarrhoea virus by TaqMan reverse transcription polymerase chain reaction. *Journal of Veterinary Diagnostic Investigation*, 14, 120–125.
- Papin J.F., Vahrson W., Dittmer D.P. (2004): SYBR green-based real time quantitative PCR assay for detection of West Nile Virus circumvents false-negative results due to strain variability. *Journal of Clinical Microbiology*, 42, 1511–1518.
- Pellerin C., van de Hurk J., Lecomte J., Tussen P. (1994): Identification of a new group of bovine viral diarrhoea virus strains associated with severe outbreaks and high mortalities. *Virology*, 203, 260–268.
- Renshaw R.W., Ray R., Dubovi E.J. (2000): Comparison of virus isolation and reverse transcription polymerase chain reaction assay for detection of bovine viral diarrhoea virus in bulk milk tank samples. *Journal of Veterinary Diagnostic Investigation*, 12, 18.
- Richards G.P., Watson M.A., Kingsley D.H. (2004): A SYBR green, real-time RT-PCR method to detect and quantitate Norwalk virus in stools. *Journal of Virological Methods*, 116, 63–73.
- Ridpath J.F., Bolin S.R., Dubovi E.J. (1994): Segregation of bovine viral diarrhoea virus into genotypes. *Virology*, 205, 66–74.
- Roeder P.L., Harkness J.W. (1986): BVD virus infection: prospects for control. *The Veterinary Record*, 119, 143–147.
- Saliki J.T., Fulton R.W., Hull S.R., Dubovi E.J. (1997): Microtiter virus isolation and enzyme immunoassays for detection of bovine viral diarrhoea virus in cattle serum. *Journal of Clinical Microbiology*, 35, 803–807.
- Varga A., James D. (2006): Real-time RT-PCR and SYBR Green I melting curve analysis for the identification of Plum pox virus strains C, EA, and W: effect of amplicon size, melt rate, and dye translocation. *Journal of Virological Methods*, 132, 146–153.
- Vilcek S., Herring A.J., Herring J.A., Nettleton P.F., Lowings J.P., Paton D.J. (1994): Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. *Archives of Virology*, 136, 309–323.
- Vilcek S., Drew T.W., McGoldrick A., Paton D.J. (1999): Genetic typing of bovine pestiviruses from England and Wales. *Veterinary Microbiology*, 69, 227–237.
- Vilcek S., Paton D.J., Durkovic B., Strojny L., Ibata G., Moussa A., Loitsch A., Rossmanith W., Vega S., Scicluna M.T., Palfi V. (2001): Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. *Archives of Virology*, 146, 99–115.
- Wittwer C.T., Herrmann M.G., Moss A.A., Rasmussen R.P. (1997): Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques*, 22, 130–138.
- Young N.J., Thomas C.J., Collins M.E., Brownlie J. (2006): Real-time RT-PCR detection of Bovine Viral Diarrhoea virus in whole blood using an external RNA reference. *Journal of Virological Methods*, 138, 218–222.
- Zipper H., Brunner H., Bernhagen J., Vitzthum F. (2004): Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination and methodological implications. *Nucleic Acids Research*, 32, e103.

Received: 2007–03–06

Accepted after corrections: 2007–05–13

## Corresponding Author:

Mgr. Eva Kosinova, Veterinary Research Institute, Department of Virology, Hudcova 70, 621 00 Brno, Czech Republic  
Tel. + 420 533 331 120, fax + 420 541 211 229, e-mail: kosinova@vri.cz