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Short communication

Real-time RT-PCR detection of Bovine Viral Diarrhoea virus in whole blood using an external RNA reference

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Received 21 March 2006; received in revised form 3 August 2006; accepted 29 August 2006
Available online 9 October 2006

Abstract

A novel two-step real-time RT-PCR assay using SYBR® Green I was developed for the detection of acute Bovine Viral Diarrhoea virus (BVDV) infection in whole blood from cattle. During infection animals experience a characteristic transient leucopenia and the number of cells per volume of blood changes over time; so quantitation of viral load by reference to a cellular housekeeping gene is not ideal as this may hide significant animal to animal variation.

Therefore, to facilitate comparison of different samples, an external RNA reference was used for normalisation whereby each sample was spiked with the RNA virus, Canine Enteric Coronavirus (CECov), prior to RNA extraction, for comparative purposes. Real-time RT-PCR was carried out with two primer sets designed to amplify either a 156 bp region of the BVDV 5′-UTR or a 280 bp region of the CECov nucleocapsid protein gene. Linearity and efficiency of the assay was established and the method assessed using samples from BVDV-challenged calves.

Viral RNA was quantified on days 6 and 14 post-challenge by real-time RT-PCR. Infectious virus isolation by traditional cell culture was negative after day 7. This study demonstrates encouraging results for rapid, sensitive and reliable detection of acute BVDV infection and provides an alternative real-time RT-PCR method for use on whole blood samples or samples where suitable housekeeping genes are not available.

Keywords: Real-time RT-PCR; Bovine Viral Diarrhoea virus; Pestivirus; Canine Enteric Coronavirus

Bovine Viral Diarrhoea virus (BVDV) is a pathogen of worldwide importance for cattle, causing significant economic losses (Brownlie, 1991). BVDV is classified within the Pestivirus genus of the Flaviviridae (Heinz et al., 2000). Pestiviruses are small, enveloped viruses that contain a single-strand positive sense RNA molecule of approximately 12.5 kb. The genome is transcribed as a single open reading frame, flanked by 5′- and 3′-untranslated regions (UTRs). The BVDV polyprotein is co- and post-translationally cleaved to give rise to a number of viral structural and non-structural proteins (Collett et al., 1988). Acute BVDV infection is often clinically unapparent or mild; clinical signs including transient pyrexia, leucopenia, ocularonasal discharge and depression. Although frequently unnoticed, acute infection can contribute to or cause a variety of problems including diseases of the reproductive, respiratory and enteric systems (Baker, 1995). BVDV infection during pregnancy may result in foetal infection. Foetuses infected before the onset of immunocompetence can become persistently infected. Persistently infected (PI) animals continuously shed virus and act as a major reservoir of infection for other cattle (Paton et al., 1999).

Current laboratory-based BVDV detection methods include serological assays, virus isolation, antigen detection and detection of viral RNA by reverse transcription (RT) PCR (Sandvik, 2005). Real-time PCR is now being used to identify, classify and quantify many viral pathogens as it is a highly sensitive and rapid method for detecting viral nucleic acid sequences in clinical specimens. Furthermore, real-time PCR is a quantitative technique and as such may be used to assess viral RNA levels. Real-time RT-PCR methods for genotyping BVDV have been described previously (Bhudevi and Weinstock, 2001, 2003; Lettellier and Kerkhofs, 2003). These papers have focused on the detection and classification of BVDV into either of the two recognised genotypes (BVDV-1 and BVDV-2) rather than monitoring the changes in viral load during the course of an acute or persistent infection. However, as real-time PCR is a quantitative technique...
technique it may also be used to assess viral RNA levels during the course of infection and recovery.

Using real-time PCR to quantify the viral RNA levels in BVDV-infected animals presents particular technical and biological challenges. Collection and processing of samples from different animals over a number of different days inevitably results in some degree of sample to sample variation in the technical detection of the target nucleic acids. This arises from differences in efficiency of RNA extraction, reverse transcription or PCR reactions which is made particularly significant by the sensitivity of this technique. These technical variations are often “controlled” for by normalisation of the signal to an internal standard, typically a “housekeeping” gene, facilitating the comparison of different data sets. This presents two further problems: firstly, whilst this is common practice, such housekeeping genes are rarely truly static and may alter under experimental conditions (Thellin et al., 1999; Whelan et al., 2003). To take account of this, best practice would recommend assessing the validity of a number of different housekeeping gene targets and comparing them under the test conditions used. This laborious task is the only way to objectively identify more stable mRNA sets. However, this process is not relevant to the BVDV situation during infection as, secondly, during acute infection with BVDV animals experience a measurable leucopenia and the number of cells per volume of blood changes over time. Traditional methods of virus isolation quantitate the amount of virus recovered from a fixed volume of blood irrespective of the cell numbers in this sample, which during acute BVDV infection will fluctuate (Polak and Zmudzinski, 2000). By standardising the viral RNA measured by real-time PCR to a housekeeping gene set the quantitation is essentially done on a per cell basis rather than a per volume basis rendering the comparison of samples from leucopenic calves more difficult and precluding a simple comparison with more traditional virological methods.

To overcome these difficulties a novel two-step real-time RT-PCR method was used with several modifications developed, which has been evaluated on samples from experimentally infected cattle. Each sample was spiked with a known amount of a second RNA virus, Canine Enteric Coronavirus (CECov), prior to RNA extraction. While the external standard made in discussion with the supplier as it enabled purification of RNA from frozen whole blood while the Viral RNA Kit was not able to process frozen blood samples. First 400 μl blood was thawed and 50 μl CECov (1 × 10^5 TCID50/ml) added as an external RNA reference. This was added to 400 μl protease solution followed by 400 μl buffer AL. After incubation at 56 °C for 10 min, the sample was cooled and 460 μl 100% ethanol added before applying to the spin column. After two washes, RNA was eluted with 50 μl tissue culture grade dH2O (Invitrogen). RNA was reverse-transcribed using Superscript II (Invitrogen) and random primers (Amersham Pharmacia Biotech).

SYBR® Green I dye was used in this real-time RT-PCR method, which binds to any double stranded DNA produced in the reaction. Each reaction contained 10 μl SYBR® Green JumpStart Taq ReadyMix for QPCR (Sigma), 2.5 μl forward primer (10 pmol/μl), 2.5 μl reverse primer (10 pmol/μl) and 5 μl template cDNA. Samples were analysed in triplicate. The PCR conditions were 94 °C for 10 min followed by 35 cycles of 94 °C for 10 s; 53 °C for 20 s; 72 °C for 20 s; 80 °C for 10 s after which a plate read was taken. The reactions were carried out on a DNA Engine Opticon 2 (MJ Research) and data analysed using the Opticon Monitor Analysis Software version 2.02. The primers were designed to amplify a 156 bp region of BVDV 5′-UTR (sense 5′-TAGTGTCAGTGTTCAAGCC; antisense 5′-CCCTGACGACCCTATCAG), or a 280 bp region of CECov nucleocapsid gene (sense 5′-CTCGGGCGGAAAGTAGTAT; antisense 5′-GCAACCCGAGMRACTCCATC) and were tested to confirm sensitivity and efficiency over a range of DNA concentrations. Primer efficiency was confirmed for each experiment using the formula:

\[
\text{Primer efficiency} = 10^{\frac{-1}{\text{slope}}} \quad (\text{Pfaffl, 2001})
\]

whereby a value of 2.0 indicates 100% efficiency. This ensured PCR products were amplified at an efficient rate and experiments were comparable. The standard samples used were plasmid DNA containing BVDV or CECov PCR products cloned into pGemT (Promega). As cDNA is a potentially more complex sample than the purified plasmid DNA used for the standard curve a test was carried out to confirm the components of a viral cDNA preparation would not be detrimental to the PCR reaction whereby standard samples were spiked with cDNA from uninfected bovine blood and the
cycle threshold (CT) values compared to non-spiked samples. Melting curve analysis of the PCR products was also carried out for each experiment and confirmed that both primer–dimers and non-specific products were absent and fluorescence was measured at temperatures where only BVDV or CECov specific amplicons were detected (data not shown). The mean CT value for CECov was determined after multiple extractions of CECov spiked blood (mean CT = 12.457, S.D. = 0.484; n = 30). Samples that fell within the range of mean CECov CT ± 1 S.D. were corrected as described in Niesters (2001); the procedure was repeated for any samples that were outside of this range.

After BVDV challenge, leucopenia was evident in all calves (Fig. 1). The decrease in white blood cell counts ranged from 7% to 52% compared to the mean pre-challenge levels for all animals 3 days post-challenge. The mean reduction was statistically significant on day 3 (P = 0.009), day 5 (P = 0.025) and day 6 (P = 0.017). This demonstrates the characteristic leucopenia observed following acute BVDV infection and highlights the complexities faced when undertaking real-time PCR on acutely infected cattle if normalizing to cellular housekeeping genes.

Primers were designed to amplify a 156 bp region of the highly conserved BVDV 5′-UTR. The linearity of the 5′-UTR assay was confirmed using cDNA obtained from BVDV spiked blood (Fig. 2A) within the range of 7000 ng to 7 ng, from samples containing $2.1 \times 10^4$ TCID$_{50}$ to $2.1 \times 10^1$ TCID$_{50}$ BVDV, indicating the sensitivity of the technique. The PCR efficiency was 2.3 and the regression coefficient was 0.993. It was not possible to efficiently detect virus in samples containing less than $2.1 \times 10^1$ TCID$_{50}$ BVDV. Linearity of the primers was...
also confirmed using plasmid DNA containing the 5′-UTR PCR product (Fig. 2B) as this was used for generation of 5′-UTR
standard curve in each experiment. The linear range of the PCR
was from $1 \times 10^3$ to $1 \times 10^6$ DNA copies/μl, with an efficiency of
2.02 and regression coefficient of 0.995. Spiking the plasmid
DNA standards with cDNA obtained from a BVDV negative
animal was not detrimental to the efficiency of the reaction
(Fig. 2C) and there was no statistically significant difference
between the CT values obtained from plasmid compared to cDNA
spiked plasmid ($P=0.997$). The linear range of the CECov primers was also assessed using cDNA obtained from samples
containing $1 \times 10^2$ to $5 \times 10^3$ TCID$_{50}$ CECov with an efficiency of
1.87 and regression coefficient of 0.990 (data not shown).

A BVDV plasmid standard curve ranging from $1 \times 10^8$ to
$1 \times 10^9$ copies/μl was used to assess 5′-UTR copy number in
each sample. The PCR efficiency for each experiment was
between 1.96 and 2.07 and the regression coefficient was greater
than 0.992 (data not shown). The 5′-UTR CT value was adjusted
accordingly based on the CT value of the CECov reaction.

By standard virus isolation one animal, calf 20, was virus
negative throughout the study period, while virus could be iso-
lated from the remaining calves for 1, 2 or 3 days between days
5 and 7 post-challenge. No virus was isolated from any animal
don day 10 or 14 post-infection (Table 1).

To validate this new methodology, two time points were chosen
for analysis: day 6 post-infection as this represents the peak
of the viraemia and also day 14 as this represents a stage when
virus isolation indicates all animals to be virus free. BVDV
viral RNA was detected in whole blood taken at days 6 and
14 post-challenge in every calf (Fig. 3). The estimates for viral
gene copy number ranged from $1.95 \times 10^3$ to $5.88 \times 10^5$, and
$1.92 \times 10^3$ to $3.22 \times 10^5$ 5′-UTR copies/ml blood on days 6 and
14, respectively.

Viral RNA copies per ml of blood were higher on day 6 than
on day 14 in three of the five calves although this difference was
only statistically significant in calf 10 ($P=0.018$). The remaining
two calves had very similar levels of viral RNA on both days
6 and 14 post-infection. Calf 20 which had been consistently
negative for infectious virus isolation was positive for viral RNA
at both time points tested.

It was to be expected that there would be some discrepancy
between the estimation of the BVDV 5′-UTR copy number of
the viral RNA and the infectious viral load in this study and
results confirm that RNA copies do not correlate with the num-
ber of infectious genomes present and therefore the potential
for infectivity and virus transmission. Lettellier and Kerkhofs
(2003) demonstrated using a BVDV type 1 virus stock that
there were 2400 copies of virus genome per infectious particle.
This presumably reflects a ratio of infectious to defective viral
genomes. Similar ratios have been observed for Yellow Fever
Virus (Bae et al., 2003). The results from the current study would
support these observations and, in vivo at least, it is proposed that
although BVDV RNA is still being detected, infectious virus has
been complexed with antibodies thereby leading to cessation of
viraemia. In the case of calf 20, virus neutralising antibody titres
of 1:564 were measured on day 14 (data not shown), supporting
this premise.

In order to compare samples it is necessary to use some form
of standardisation and often housekeeping gene expression is
used to achieve this. However, housekeeping gene expression
can vary and the use of these genes as internal controls should
be carefully considered in relation to cell type and the cell
metabolism (Thellin et al., 1999). The large number of pub-
llications focusing on the validation of internal reference genes
demonstrates the difficulty in selecting appropriate candidates.
Indeed, many authors now recommend the use of at least two
(Dent et al., 1997) or three (Bustin, 2000; Vandesompele et al.,
2002) validated housekeeping genes to ensure the accuracy of
data. In addition, the use of housekeeping genes to control for
the variation in RNA extraction and cDNA synthesis is diffi-
cult when a fixed amount of blood is analysed (Johansson et
al., 2000). The normal range of leucocyte counts in blood from
healthy cattle is $4–12 \times 10^9$ l$^{-1}$ (Blood, 1994), thus demonstrat-
ing a potential threefold variation in cell numbers. Furthermore,
acute BVDV infection causes transient leucopenia, leading to
a reduction in numbers of circulating leucocytes which can be
pronounced. Virus virulence as well as host factors may result in
the reduction of white blood cells ranging from mild to severe.
In the current study all five calves were challenged with the same
dose of virus and the degree of leucopenia varied from animal
to animal (Fig. 1).

To facilitate virus quantification per fixed volume of blood
in the face of characteristic leucopenia observed in cattle during
acute BVDV infection, a novel two-step real-time RT-PCR

\begin{table}[h]
\centering
\caption{Virus isolation following challenge}
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Calf} & \textbf{Day post-challenge} & 3 & 5 & 6 & 7 & 10 & 14 \\
\hline
1 & - & - & + & + & - & - \\
3 & - & - & + & + & - & - \\
10 & - & + & + & + & - & - \\
15 & - & - & + & - & - & - \\
20 & - & - & - & - & - & - \\
\hline
\end{tabular}
\footnotesize{Virus isolation from buffy coat was performed by immunofluorescence assay after two passages in FBL cells.}
\end{table}

Fig. 3. Quantification of BVDV RNA. Mean BVDV RNA copies/ml blood from calves on day 6 (●) and day 14 (□) post-challenge. Error bars show standard
error of the mean * indicates significant values.

Table 1
Virus isolation following challenge

| Calf | Day post-challenge |
|------|-------------------|
| 1    | 3 5 6 7 10 14     |
| 3    | - - + - - -       |
| 10   | - + + + - -       |
| 15   | - - + - - -       |
| 20   | - - - - - -       |

Virus isolation from buffy coat was performed by immunofluorescence assay after two passages in FBL cells.
method which utilised an external RNA reference standard was developed. A second RNA virus, Canine Enteric Coronavirus (CECov) was used as a control for the each stage of the method. To enable ease of sample handling the method was based on quantification of BVDV RNA per volume of frozen whole blood. Each bovine blood sample was spiked with a known amount of the reference CECov prior to RNA extraction. The coronavirus was co-extracted and subsequently amplified in a quantitative manner. This enabled validation of nucleic acid isolation and amplification efficiency for each sample and detection of those samples for which sample preparation or amplification failed by determining the signal generated from the CECov standard. This system of ‘spiking’ samples with a heterologous virus has been used previously for Hepatitis C virus quantification (Cleland et al., 1999; Castelain et al., 2004) and for the detection of Epstein–Bars virus and Cytomegalovirus (Niesters, 2001).

Currently there is much interest in control strategies for BVDV infection of cattle. To this end rapid and reliable diagnosis of both persistently and acutely infected cattle is imperative. Molecular diagnostic methods are being increasingly utilised as tools for the detection of numerous viral pathogens. The use of real-time RT-PCR methods to establish the presence or absence of BVDV RNA in cattle may have important implications for future diagnostic screening and control strategies.

Acknowledgement

The authors wish to thank Dr. Kerstin Erles (RVC) for kindly donating the Canine Enteric Coronavirus and corresponding primer sequences.

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