Full Length Research Paper

Establishment of EvaGreen qPCR for detecting bovine rotavirus based on VP7 gene

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The aim of the present study was to establish a method of EvaGreen real time fluorescence quantitative polymerase chain reaction (qPCR) for detecting quickly bovine rotavirus (BRV) in fecal samples from calves with diarrhea. The specific primers were designed and synthesized according to BRV VP7 gene in Genbank. VP7 gene was cloned into the pMD18-T vector. The bovine viral diarrhea virus, bovine coronavirus, porcine epidemic diarrhea virus, bovine mycobacterium tuberculosis and negative control were detected with qPCR. Plasmids in five 10-fold gradients were detected using qPCR. The results show that a EvaGreen qPCR was established in the present study. Only BRV displayed amplification curve; cross-reactivity between BRV and the other viruses or bacteria was not observed. The amplification curve of pMD18-T vector plasmid (diluted in 10⁻¹ to 10³ gradient) was typical S shape. The detection limit of qPCR was 8.0 copies/μL, namely 10⁰ plasmid concentrations. The coefficients of variation in both intra-assay and inter-assay was less than 2%. The established EvaGreen qPCR had a high specificity, sensitivity and reproducibility. It can be applied to clinical diagnosis and epidemiological surveys of BRV.

Key words: Rotavirus, VP7 gene, evagreen, real-time quantitative PCR, bovine.

INTRODUCTION

Neonatal calf diarrhea is a common disease affecting the newborn calf worldwide, threatening the cattle production along with significant morbidity and mortality and inducing severe economic losses (Wei, 2011). Group A rotaviruses (RVA) are known to be important viral diarrheal agents in infants and young animals, including calves.

The numbers of the rotavirus-associated mortality are estimated to be 453,000 in 2008 (Tate, 2012; 2013). The numbers of deaths is particularly high in developing countries (Abe et al., 2009). Even in developed countries, rotavirus remains an important cause of morbidity.

Rotaviruses possess 11 segments of double-stranded ribonucleic acid (dsRNA) and two outer capsid proteins: VP4 (encoded by gene segment 4) and VP7 (encoded by gene segment 7, 8 or 9 depending on the strain), both of which are independently responsible for virus neutralization (Estes, 2001). RVA strains are antigenically heterogeneous, and are classified in multiple G and P types de-
fined by VP7 and VP4 outer capsid proteins (Papp et al., 2013). The neutralization specificity related to VP7 is referred to as the G serotype (for glycoprotein), and that associated with VP4 is referred to as the P serotype (for protease-sensitive protein) (Anthony et al. 1999). Currently, group A rotavirus has been classified into at least 15 G serotypes (G1-G15) and 21 P genotypes (P1-P24) (Ram, 2004). With regard to bovine, at least nine G serotypes (G1-4, 6-8, 10, 11) and three P genotypes have been found so far (Santos and Hoshino, 2005; Papp et al., 2013). Feray et al. (2010) reported G6 was the predominant G-type, detected in 40/53 samples (75.4%), while P[11] was the predominant P-type, detected in 52/53 samples (98.1%). The most common VP7/VP4 combinations were G6P[11] (60.3%) and G10P[11] (24.5%). A similar finding was reported by Swiatek et al. (2010). Our study found that G6 and G10 serotypes were 29 (54.7%) and 8 (15.1%) in positive samples for VP7. The main combinations of BRV G serotype and P genotype were G6P[5] (28.3%) (Wei et al., 2013).

VP7 and VP4 proteins elicit the production of neutralizing antibodies, define the antigenic specificities, referred to as G type and P type, respectively, and are the major antigens neutralizing immune responses during rotavirus infections (Aminu et al., 2010). Rotavirus VP7 gene is highly conservative at both ends of open reading frame (ORF), such that it is feasible to detect rotavirus serotypes utilizing the rotavirus specific primers. VP7 has been shown to be involved in the early interactions with cell-surface molecules, during the rotavirus entry process (Lopez and Arias, 2006; Martha et al., 2012). Moreover, PCR can be used to detect rotavirus VP7 not only stool specimens also cerebrospinal fluids and sera (Hiroshi et al., 1994).

Currently many methods (including polymerase chain reaction, PCR) are available for detecting rotavirus, especially VP6 antigen (Luan et al., 2006; Gutierrez-Aguirre et al., 2008; Zhu et al., 2011; Fan et al., 2011). Unfortunately, PCR assays require sophisticated equipment, which is costly to maintain, and must be performed in specialized laboratories (Xie et al., 2012). So far, little information regarding real-time quantitative PCR (qPCR) utilized to detect RV VP7 antigen has been known. The real-time quantitative PCR (qPCR) methods are not only fast and accurate, and also can test against different target sequences. Its specificity is very high (Okada and Matsumoto, 2002; Santos and Hoshino, 2005). Quantitative PCR (qPCR), also known as real-time PCR, has become a powerful tool for the amplification, identification and quantification of nucleic acids. Its ability to quantitatively and specifically detect genes has been invaluable for both research and diagnostic applications (Schweitzer and Kingsmore, 2001). The qPCR using a simple DNA dye is a popular choice among academic laboratories for PCR experiments (Bustin, 2002). Compared to conventional reverse transcript PCR (RT-PCR), qPCR has been shown to be more rapid and more sensitive for the detection and quantification of rotavirus (Mackay et al., 2002; Kang et al., 2004; Pang et al., 2004). EvaGreen (EG) is a novel DNA-binding dye. It has been reported to be used for DNA quantification, DNA conformation detection quantitative PCR (Ihrig et al., 2006).

Currently, very little research of EvaGreen Real-time Quantitative PCR (qPCR) is performed for detection of BRV (Ihrig et al., 2006). Currently, very little research of EvaGreen Real-time Quantitative PCR (qPCR) has been performed for detection of bovine rotavirus (BRV) (Gouvea et al., 1990; Mohan et al., 2006). It has not been applied in detecting bovine rotavirus in China. The aim of the present study was to establish EvaGreen Real-time Quantitative PCR (qPCR) for detecting BRV VP7 gene in fecal samples.

MATERIALS AND METHODS

Fecal samples

Fecal samples were collected from 62 calves with diarrhea that were one to thirty days old and located on dairy farms in Lanzhou (26 samples), Qingyang cities (19 samples) and Gannan autonomous prefecture (17 samples) of Gansu province of China, from November 2010 to April 2011. TRizol (a nucleic acid extraction reagent; Invitrogen, Beijing, China) was added to the collection tube in advance. All fecal specimens were stored at -20°C until further use. A 10% suspension of each fecal sample was prepared in phosphate buffered saline (PBS), pH 7.2 and centrifuged at 4,000 rpm until 15 min at 4°C. The supernatants of 62 fecal samples were subjected to ELISA for detecting the presence of RV with commercially rotavirus detection kits (Lanzhou Institute of Biological Products Company, Lanzhou, China) and following the protocol of the manufacture instructions. The results were interpreted using the OD values obtained at 450 nm with ELISA reader (BioTec, Dresden, Germany). All positive samples from ELISA were used for the subsequent experiments. Fecal supernatants were stored at 4°C.

Primers designs and synthesis

For qPCR, based on the deposited genome sequences of BRV VP7 in GenBank (accession number No. GO433985), specific primers were designed using Primer Premier 5.0 software according to the highly conserved regions:

**Forward:** 5'-GATGGTATTGAATACAC-3’ (nts 51-71 of GO433985).
**Reverse:** 5'-GACCTGTGGCCCATCC-3’ (nts 376-392 of GO433985).

The length of predicting product is 342 bp. The concentrations of primers (100, 200, 300 and 500 nM) were evaluated. The formation of primer-dimers was assessed by melting curve analysis.

Thus, only those concentrations of primers that showed dimer-free reactions were used for the final analysis. Primers were synthesized from Takara Bio, Dalian, China.

Cell cultures

The neonatal calf diarrhea virus (NCDV) strain (AV-51, purchased from Chinese Veterinary Drugs Supervisor Institute, Beijing, China) and ELISA positive fecal samples were inoculated to single layer MA-104 cells as described previously (Wei et al., 2010). The cells
were cultured for 3-4 days at 37°C. The process ended when the cytopathogenic effect (CPE) was higher than 90%. Then, the cells were frozen and thawed 2 to 3 times. The viral supernatant was collected for RNA extraction or stored at -80°C until processed.

RNA extraction and cDNA synthesis

According to the manufacturer’s instructions, total RNA was extracted from the viral supernatants of the neonatal calf diarrhea virus (NCDV) strain (AV-51) and fecal samples using the TRizol RNA extraction kit (Invitrogen, Beijing, China). Briefly, PCR was performed in a 25 μL volumes that included 15.5 μL diethylpyrocarbonate (DEPC) water, 0.5 μL (10 mM) deoxyribonucleotide triphosphate (dNTPs), 2.5 μL 10×PCR buffer, 0.5 μL Tag enzyme. 0.5 μL BRV primer, 0.5 μL BRVR and 5 μL cDNA. The PCR products were electrophoresed on 1.5% agarose gel (Amresco, USA) containing 1×Gel Red (BIOTIUM, Hayward, CA, U.S.A.) and subsequently analyzed with the software CS Analyzer Ver 3.0 (ATTO, Tokyo, Japan).

The cDNA was synthesized from the NCDV strain and extracted viral RNA by reverse transcription reaction and used for PCR amplification of the VP7 gene. The expected amplicons were 342 bp sizes.

Preparation of plasmid standard template

The amplified VP7 cDNA fragments were cloned into pMD18-T vectors. Then, pMD18-T vectors were sequenced and validated. The positive plasmids were quantitatively measured using the optimized reaction conditions to establish a standard curve with the logarithm values of template starting copy number as the horizontal axis and Ct values as the vertical axis. The positive plasmids were used as standards (pMD-VP7), which were serially diluted 10-fold gradients to 4.0×10^3, 4.0×10^2, 4.0×10^1 and 4.0×10^0 copies/μL.

EvaGreen Real-time Quantitative PCR (qPCR)

The qPCR was performed in a 50 μL reaction systems, which consisted of 25 μL EvaGreen qPCR Master Mix (Chaoshi biotechnology company, Shanghai, China), 1 μL BRV primer (10 μM), 1 μL BRVF primer (10 μM), 5 μL cDNA and 18 μL deionized water. The experiment Detector was set to SYBR, and the Quencher was set to None. The conditions for qPCR were as follows: initial denaturation at 95°C for 15 min followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 72°C for 40 s, and a final extension at 72°C for 10 min. The qPCR was done in ABI quantitative PCR instrument (ABI PRISM 7300 type, Applied Biosystems incorporation, USA).

Establishment of the standard curve for qPCR

The 10-fold diluted plasmid standards (4.0×10^1, 4.0×10^2, 4.0×10^3, and 4.0×10^4 copies/μL) were detected quantitatively using qPCR assay. Such the standard curve had been established with the logarithmic values of the starting copies as the horizontal axis (X) and Ct values as the vertical axis (Y).

Specificity tests

To examine the analytical sensitivity, the RNA was extracted from the viral supernatants of BRV stools, bovine viral diarrhea virus (BVDV), bovine coronavirus (BCV), porcine epidemic diarrhea virus (PEDV) and bovine mycobacterium tuberculosis (provided by Lanzhou Veterinary Research Institute, Chinese Academy of agricultural sciences, Lanzhou, China), respectively. RNA was reverse transcribed. RNA templates were amplified with the established qPCR. Meanwhile, the negative control was detected. Meanwhile, the negative control was set.

Sensitivity tests

The plasmids were diluted on the serial 10-fold dilutions (10^3, 10^2, 10^1, 10^0 and 10^−1 gradients) and detected with the established qPCR to evaluate the sensitivity of qPCR.

Stability

For the intra-assay variability, the plasmids in 5 dilution gradients (10^3, 10^2, 10^1 and 10^0) were detected using qPCR. The test was repeated thrice. The copy numbers, mean, standard deviation and variation coefficient (CV) were calculated from the standard curve for each dilution gradients.

For inter-assay variability, the plasmids in 5 dilution gradients were detected with the qPCR for three times every three days. The copy numbers, average, standard deviation and variation coefficient were also calculated as the methods mentioned above.

Fecal sample detection

To evaluate the diagnostic efficacy of qPCR assay, cDNAs of 62 fecal samples were detected using the established qPCR. The findings were compared with that of ELISA method to verify the coincidence rate of two methods.

RESULTS

Screening of fecal samples by ELISA

The screening of 10% suspension of 62 fecal samples indicated that 12 fecal samples were positive for BRV, with a prevalence rate of 19.36%.

Amplification of BRV VP7 gene

As shown in Figure 1, a predicted 342 bp band was found in agarose gel electrophoresis. The result testified they are group A BRV.

Dynamics curve and the standard curve of qPCR

The reaction conditions of qPCR were optimized. The optimum reaction conditions were as follows: initial denaturation for 15 min at 95°C, denaturation for 15 s at 95°C, annealing for 30 s at 55°C, elongation for 40 s at 72°C, by 40 cycles, and a final elongation of 10 min at 72°C.

The dynamics curve of qPCR was acquired (Figure 2). The recombinant plasmids (pMD-VP7) in five 10-fold gradients from 4.0×10^1 to 4.0×10^3 were detected using the optimized qPCR reactions, respectively. Ct values were...
So far, it has not been observed. The reactivity between BRV and other viruses or bacteria was only BRV displayed amplification curve, the c dynamics helped the specificity of the assay. The predicted 342 bp band was found. 1: blank control; 2 and 3: extracted total RNA from fecal samples; 4: marker.

![Amplification of BRV in agarose gel electrophoresis](image)

The predicted 342 bp band was found. 1: blank control; 2 and 3: extracted total RNA from fecal samples; 4: marker.

![Amplification Curve](image)

The dynamics curve of qPCR.

28.55, 24.42, 20.67, 17.42 and 14.48 respectively. Such, the standard curve was established (Figure 3). The slope, intercept and correlation coefficient ($R^2$) of the standard curve were -3.574, 31.77 and 0.997 respectively, indicating a strong linear relationship. Gene amplification efficiency ($E$) equalled to (1.91 - 3.574) x100% = 91%. Such, the regression equation was expressed as $Y=-3.574 \log X +31.77$. It can be calculated that $Ct$ value equated to 31.77-3.574X.

**Specificity test results**

As can be seen from Figure 4, the optimized rotavirus qPCR assay was tested against other viruses and bacteria, which included BCoV, PEDV and MB, in order to demonstrate the specificity of the assay. The results show only BRV displayed amplification curve, the cross-reactivity between BRV and other viruses or bacteria was not observed. It indicated the established qPCR assay had an excellent specificity. It can be applied to detect diarrhea sample clinically.

**Sensitivity test results**

The plasmids in five dilution gradients were detected with the established qPCR (Figure 5).

The amplification curve of pMD-VP7 plasmid (diluted in $10^1 \sim 10^7$ gradient) was typical S shape with an excellent appearance. Ct values were 28.55, 24.42, 2.067, 17.42 and 14.48, respectively. The corresponding copy numbers were $1.80 \times 10^3$, $4.11 \times 10^2$, $6.21 \times 10^1$, $8.03 \times 10^0$ and $9.68 \times 10^{-1}$, respectively. Therefore, the detection limit of qPCR was $8.0 \text{copies/μL}$, namely $10^3$ plasmid concentration.

**Stability test results**

As shown in Table 1, the variation coefficients (CV) of intra-assay reproducibility and inter-assay reproducibility were 1.0-1.1 and 1.7-2.0, respectively, which demonstrated the qPCR assay was stable and reliable.

**Detection results of fecal samples**

Detection results of fecal samples showed that 10 (all of which were positive for ELISA) out of 62 fecal samples were found positive for BRV by qPCR. The coincidence rate of qPCR and ELISA was 83.3%. qPCR had a higher sensitivity and specificity.

**DISCUSSION**

Real-time PCR can be carried out using either probes or DNA dyes (Higuchi et al., 1993; Wilhelm and Pingoud, 2003; Anne, 2011). SYBR Green exhibits a very strong fluorescent signal, but it has been shown to inhibit the PCR reaction and has a narrow dynamic range and lower reproducibility than other detection chemistries (Gudnason et al., 2007). EvaGreen is another DNA dye which is less inhibitory to PCR than SYBR Green and is marketed as an alternative. EvaGreen dye performed better than SYBR Green in general, and high reaction efficiencies can be achieved using the dye. In cases where template sequence tends to vary, dye-based detection helps prevent false negatives that might result from base pair mismatches in a sequence-specific probe binding region (Anderson et al., 2003; Papin et al., 2004). It has recently been used for quantitative real-time PCR (qPCR), post-PCR DNA melt curve analysis and several other applications (Mao et al., 2007). So far, it has not been reported that qPCR is utilized to detect bovine rotavirus in China. The present study was to develop a higher specificity and sensitivity method for detecting BRV VP7 gene in fecal samples using EvaGreen dye.
Figure 3. The standard curve of qPCR. The slope, intercept and correlation coefficient ($R^2$) of the standard curve were -3.574, 31.77 and 0.997 respectively, indicating a strong linear relationship.

Figure 4. Specificity of qPCR assay. BRV displayed amplification curve, the cross-reactions between bovine rotavirus and other viruses: BVDV, bovine viral diarrhea virus; BcOv, bovine coronavirus; PEDVPEDV, porcine epidemic diarrhea virus or bacteria bovine; MB, mycobacterium tuberculosis and negative control were not observed, indicating a high specificity for qPCR assay.

Figure 5. Sensitivity of qPCR assay. M, 100bp DNA Marker; 1-2, padding and negative control; 3-7, the 10-fold serial plasmid dilutions ($10^3$, $10^2$, $10^1$, $10^0$ and $10^{-1}$ gradients).
Table 1. Intra-assay and Inter-assay reproducibility test of qRT-PCR.

<table>
<thead>
<tr>
<th>Dilution gradient of Standard plasmid</th>
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<th>Intra-assay reproducibility</th>
<th>Inter-assay reproducibility</th>
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<tr>
<td></td>
<td></td>
<td>X ± SD</td>
<td>CV(%)</td>
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<tr>
<td></td>
<td>10^1</td>
<td>3</td>
<td>15.1±0.6</td>
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<tr>
<td></td>
<td>10^2</td>
<td>3</td>
<td>18.4±0.7</td>
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<tr>
<td></td>
<td>10^3</td>
<td>3</td>
<td>21.5±0.6</td>
</tr>
<tr>
<td></td>
<td>10^4</td>
<td>3</td>
<td>26.1±0.7</td>
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Note: * Copy µL

In the present research, a 342 bp VP7 gene of BRV was amplified from fecal samples with RT-PCR. qPCR has been developed for determining BRV VP7 gene. The quantities of RNA in qPCR ranged between 8.03×10^0 and 1.80×10^3 copies per reaction. The established qPCR was high specificity, sensitivity and stability. However, because of inadequate conditions and detection of small samples, the specificity of the experimental methods and result repetition need to be improved in the future. In addition, BRV Kit has not been developed (Luan et al., 2006; Wei et al., 2010). The human ELISA Kit was used in this study. The coincidence rate of qPCR and ELISA in our findings needs to be investigated further.

It is concluded that the qPCR using EvaGreen dye is a rapid, sensitive and stable method for the detection of BRV VP7 gene. The qPCR has a low risk of contamination and is less time and manpower consuming than conventional RT-PCR. It could be used for clinical diagnosis and epidemiological survey of bovine rotavirus.

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REFERENCES

Martha NC, Fanny G, Orlando A, Carlos AG (2012). Rotavirus VP4 and VP7-Derived Synthetic Peptides as Potential Substrates of Protein


