Quantitative Detection of Group A Rotavirus in Humans and Animals by Real-Time Reverse Transcription-Polymerase Chain Reaction

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ABSTRACT

The detection as well as quantification of Group A rotavirus in the diarrhoeic faecal samples from humans and animals was carried out by real time reverse transcription PCR assay (qRT-PCR) by using primers from the conserved region of gene segment 9 in Group A rotaviruses. The standardized test detected Group A rotaviruses in the range of $1.70 \times 10^5$ to $1.28 \times 10^5$ copies in humans, $2.21 \times 10^3$ to $4.78 \times 10^4$ copies in calves, $1.29 \times 10^2$ to $2.03 \times 10^4$ copies in kids and $7.21 \times 10^3$ to $3.52 \times 10^4$ copies/reaction in lambs.

Keywords: Diarrhoea, Group A Rotavirus, RT-PCR, real-time reverse-transcription PCR

Introduction

Rotaviruses are double-stranded RNA viruses, classified into seven different serogroups (A-G), based on the antigenic specificity of the middle layer protein of the virus as well as on the pattern of electrophoretic mobility of the 11 RNA segments of the viral genome (Estes, 1996). Of the seven serogroups, Group A, B, and C are known to cause gastrointestinal infections in humans and animals. Group A viruses are predominant and most commonly associated with severe, dehydrating diarrhoea in children and young animals worldwide. The outcome of infection is more serious in developing countries where an estimated 600,000 deaths occur annually in children less than five years of age (Parashar et al., 1998). In India, one out of every 250 children or about 100,000–150,000 children die of rotavirus diarrhoea each year, accounting for 17% of world’s estimated deaths (Broor et al., 2003). Likewise, heavy losses as a result of rotavirus infections in animal husbandry industries are well documented, with the incidence of rotavirus associated diarrhoea in calves below one month of age ranging between 10% and 52% (Singh and Pandey, 1990).

Several molecular techniques have been exploited for the development of highly sensitive and rapid assays for the detection of Group A rotaviruses. Rotaviruses are usually detectable by enzyme immunoassays (EIA) for up to one week after infection or for prolonged periods in immunocompromised patients. The enzyme immunoassays employ antibodies specific for Group A-VP6 antigen in faecal samples. However, reverse transcription-PCR (RT-PCR) has reportedly increased the detection rate of rotavirus A by up to 48% compared to EIA or electron microscopy (Pang et al., 1999; Gunson et al., 2003). In infants with severe rotavirus diarrhoea, the virus is detectable by RT-PCR for up to 57 days after hospital admission (Richardson et al., 1998). Recently, real-time PCR
assays with high sensitivity and specificity have been developed. These assays have an edge over the conventional RT-PCR as they help to correlate the severity of clinical disease with the level of viral replication. In this study, we report the development of a real time PCR based method for the rapid, specific and quantitative detection of Group A rotavirus from diarrhoeic samples of children and animals suffering from gastroenteritis.

Materials and Methods

Specimen collection

A total of 30 stool samples were collected from children (under 5 years of age) exhibiting symptoms of diarrhoea from private pediatric hospitals in Bareilly, Uttar Pradesh. Five stool samples were collected from pediatric patients not exhibiting symptoms of gastroenteritis and used as healthy controls for the purpose of this study. Similarly, a total of 30 faecal samples were collected from diarrhoeal cattle/buffalo calves (23), and goat kids (4) and lambs (3) from organized farms in Bareilly, Uttar Pradesh and Avikanagar, Rajasthan, respectively, and five non-diarrhoeal samples were used as controls. The samples were collected from the period of December to February, 2011 and stored at -20°C until analyzed.

Nucleic acid extraction from stool samples

Rotaviral dsRNA extraction was accomplished with TRIzol reagent as per the manufacturer’s instructions using 50-100 mg of fresh or frozen faecal sample.

Preparation of standard plasmid DNA

The RT-PCR was put for the known positive Group A rotavirus strain available in the Division of Veterinary Public Health, IVRI, using the forward primer; 5’-GAT CCG AAT GGT TGT GTA ATC CAA T-3’, nt 531 to 550 and reverse primer; 5’-AAT TCG CTA CGT TTT CTC TTG G-3’, nt 824 to 808 from the region of gene segment 9 which is conserved in all Group A rotaviruses, with amplification products of 304 bp in length. In order to obtain purified PCR amplicons for cloning, the specific gene amplified products were gel eluted using QIA quick gel extraction kit (Qiagen, Germany) as per the manufacturer’s instructions. The standard plasmid was constructed by inserting the PCR fragment (304bp) into a pGEM-T Easy vector according to the manufacturer’s instructions (Promega, Madison, WI, USA). The recombinant plasmid DNA was characterized using restriction endonucleases, namely Apal and PstI, and then stored at -20°C until further analysis.

qRT-PCR for detection and quantification of Group A rotavirus

The detection as well as quantification of rotaviral load in the diarrhoeic faecal samples from humans and animals was carried out by SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR kit (Invitrogen, USA) on Mx3000P Real-Time PCR System (Stratagene, USA) operated by MxPro™ QPCR software. The assay was carried out by using the primers as described above. The qRT-PCR reaction was run in duplicates, each in a final volume of 20 µl, comprising of the following components: 2x SYBR Green reaction mix 10 µl, forward primer 10 pmol/µl, reverse primer 10 pmol/µl, SuperScript III RT/ Platinum Taq Mix (0.4 µl), template RNA (1.0 µl) and 7.0 µl of nuclease free water. The cycling conditions included an initial step for cDNA synthesis at 50°C for 3 min (segment I), followed by denaturation at 95°C for 5 min (segment II), 40 cycles each of denaturation at 94°C for 15 sec, annealing and extension at 60°C for 30 sec (segment III), 40°C for one min (segment IV) and, dissociation (melting) curve consisting of 95°C for 30 sec, followed by 61°C for 30 sec and gradual increment from 61°C to 95°C @ 2 degree per min and lastly 95°C for 30 sec (segment V). The fluorescence was recorded at the end point of each of the 40 cycles. After the qRT-PCR was completed, the amplicons of each sample were run in 2% agarose gels and visualized on a UV transilluminator.

Determination of initial copy number

In order to get PCR efficiency and slope value, a standard curve method was employed using SYBR Green chemistry. The concentration of the standard linearized plasmid containing the 304 bp insert of VP7 gene strain of Group A rotavirus was measured
by Nanodrop (USA) and converted to genome copy numbers by using the molecular weight of DNA to construct a standard curve. The genome copy number per microlitre was determined as per the methodology of Adams (2006). The plasmid DNA copies worked out in the known concentration of standard plasmid was then diluted ten folds. The standard curve in terms of a regression line equation was drawn by plotting the known log copy number in the dilutions against the threshold values ("Ct"). The PCR efficiency and slope value were calculated as per default setting of machine.

Detection and quantification of Group A rotavirus in clinical samples

The standardized qRT-PCR was used for detection as well as quantification of group A rotavirus genome copy numbers in diarrhoeal samples from humans and different animals species. Each assay was performed in duplicate and each run included two negative controls. The genome copy numbers per microlitre of the samples were estimated based on the regression line equation of standard curve, by using the formula, Log10 copy number = Ct – y intercept/slope (Adams, 2006).

Results

Preparation of standard plasmid DNA

The purified PCR product of 304 bp size was successfully cloned in pGEM-T Easy vector for the construction of standard plasmid DNA and confirmed by the release of insert from the plasmid after digestion with ApaI and PstI restriction enzymes (Fig. 1).

Standardization of the assay

The standardized qRT-PCR assay allowed the amplification of a 304 bp product in the region corresponding to VP7 gene of Group A rotavirus. The absence of any primer-dimer in the agarose gel indicated that the fluorescence from intercalated SYBR Green dye was obtained from the specific amplified product. The amplification plots and dissociation curves from the plasmid DNA served as a base for detecting Group A rotavirus and its quantification (copy number) in the clinical samples from positive cases. To check the assay specificity, melting point analyses was performed. The Tm value was recorded as 81.3°C after completion of the dissociation (melting) curve.

Interpretation of copy number

To quantify the copy numbers contained in the samples, a standard curve was created with 10-fold dilutions of plasmid DNA (concentration, 6183 ng/µl) containing the target gene. On plotting the known plasmid DNA copy numbers in serial ten-fold dilutions (10⁸ to 10⁴ copies) against the corresponding average Ct (threshold cycle) values i.e., 10.35, 13.27, 16.68, 20.25 and 23.45, respectively, a standard curve was obtained that served as a basis for estimating the number of genome copies of rotavirus in the positive clinical samples (Fig. 2). Linear regression of the Ct values and the quantity of plasmid DNA revealed a good negative linearity (r² = 0.99, error = 0.2424, slope = -3.31, and y-intercept = 36.71).

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The rotaviral load in terms of copy numbers per microlitre of the sample was calculated based on the extrapolation of the Ct values of the samples against the standard curve (Fig. 3, 4). With 100 ng of stool used in each qRT-PCR reaction, Group A rotavirus was detected in the range of 1.70x10¹ to 1.28x10⁵ copies in 24 of diarrhoeic stool samples. None of the healthy controls were detected positive.

Detection and quantification of Group A rotavirus in animals

qRT-PCR testing of diarrhoeic faecal samples from 30 animals (calves-23, goat kids-4 and lambs-3) revealed positivity for rotavirus in 25 samples. With 100 ng of faecal sample used in each qRT-PCR reaction, Group A rotavirus was detected in the range of 2.21x10³ to 4.78x10⁴ copies in bovine, 1.29x10² to 2.03x10⁴ copies in caprine and 7.21x10³ to 3.52x10⁴ copies in ovine diarrhoeal samples.

Discussion

The qRT-PCR systems are slowly replacing the less sensitive conventional RT-PCR assays and
electropherotyping by RNA-PAGE as they allow for the detection as well as simultaneous quantification of RNA viruses in research and diagnostic laboratories. SYBR Green I dye binds to double-stranded nucleic acids generated during the amplification, providing sensitive detection of products in real time (Pang et al., 2004). The amplification efficiency and linearity range of the qRT-PCR assay developed was demonstrated by amplifying duplicate aliquots of 10-fold serial dilutions (10^8 to 10 copies) of the appropriate plasmid DNA standard, cloned in pGEM-T Easy
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vector. Standard curve with 10-fold serial dilutions of plasmid DNA controls (from 10^2 to 10^4 copies) was prepared and assayed. The resulting standard curve with slope value -3.31 and Y intercept value as 36.71; with strong correlation coefficients (r² = 0.99), indicated strong linear relationships over the log copy number range examined. PCR amplification efficiency for each assay was calculated from the slope of the standard curve as 100.9%, which was in agreement with the published reports, wherein a perfect assay should have a y intercept between 33 and 37 cycles, r² of 1.00 and a slope of -3.32 (100% efficiency) (Adams, 2006).

Various workers have used SYBR green in the amplification reaction to monitor PCR product accumulation in each cycle and for quantification of rotaviral load from diarrhoeic samples, as there is a direct relationship between the number of PCR cycles that are required to detect a template and the number of template molecules present initially in the sample (Schwarz et al., 2002; Logan et al., 2006). Serial 10-fold dilutions of the plasmid containing the 379 bp product insert derived were used for calculating the amount of rotavirus RNA present in the diarrhoeic samples by Kang et al. (2004).

In the present study, Group A rotavirus was detected in 24 human diarrhoeic samples out of 30 tested, in the range of 1.70x10^5 to 1.28x10^6 copies in 100ng of the stool samples. In another study, the quantities of rotavirus RNA ranged between 1.70x10^5 and 9.23x10^5 RNA copies per reaction (Zeng et al., 2004). Likewise, Schwarz et al. (2002) have reported the detection of as low as 10 RNA molecules of viral RNA of Group A rotaviruses in clinical samples obtained from various animal species and human beings by fluorimetry using the SYBR Green I dye in qRT-PCR assay.

The testing of diarrhoeic faecal samples from 30 animals (calves-23, goat kids-4 and lambs-3), revealed positivity for rotavirus in 25 samples. The results are in agreement with published reports which have described qRT-PCR to be rapid and more sensitive for the detection and quantitation of rotavirus compared to conventional RT-PCR assay (Gutierrez-Aguirre et al., 2008). However, the data pertaining to quantification of Group A rotavirus in faecal samples from calves, kids and lambs could not be compared with for the want of any published work of this nature.

Thus, the present study describes the development of qRT-PCR assays with high levels of sensitivity and specificity for the detection and quantification of Group A rotaviral particles in stool samples.

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References


