INTRODUCTION

Enteric and respiratory viral infections are among the most common worldwide problems and are being recognized as the major challenge to the livestock sector (Razzaque et al., 2009; Lorenz et al., 2011). Among several causes of acute viral gastroenteritis, rotavirus (RV), a member of the Reoviridae family, is the most prominent cause of neonatal diarrhea worldwide (Estes and Kapikian, 2007), causing up to 25% mortality in young animals (Dhama et al., 2009). Losses assimilate to the farmers through additional expense for enhanced managemental issues, medicines, reduction in optimum animal growth and loss of the calves (Razzaque et al., 2009). RVs have been classified in 8 species (A–H) (Matthijnssens et al., 2012), where species A rotaviruses (RVA) are predominantly present in humans, bovines and other mammalian and avian species throughout the world (Matthijnssens et al., 2012). There are six structural proteins (VP1 to VP4, VP6 and VP7) and six non-structural proteins (NSP1 to NSP6) of which NSP4 has pleiotropic role and has been a target for RV diagnosis (Ciarlet et al., 2000; Estes and Kapikian, 2007).

Rotaviruses of animal origin have shown established potential for interspecies transmission and additionally impose risk for human infection, signifying need for rapid and sensitive method for better case management and detection of RVs early in the course of infection. The common methods...
Real-time PCR assays have been described for detection of human RVs (Zeng et al., 2008) and reports are limited for animals RVs and in particular from India are not available for the use of the said test for detection of RVs in bovines. In the present study, we describe the development and application of a specific SYBR Green based real-time PCR with subsequent melting curve analysis for detection of RVA using a primer set specific to genome segment 10 (NSP4) of bovine RVA strains. As the primer binding site of RVA using a primer set specific to genome segment 10 (NSP4) of bovine RVA strains. As the primer binding site (Esly et al., 2006). Among the several chemistries available for real-time PCR assays, SYBR Green based assays are most widely used. Recently, real-time PCR based methods have been reported for detection of many different viruses of veterinary importance such as Foot and mouth disease virus, bluetongue, Newcastle disease virus, avian influenza virus (Aguero et al., 2007).

**MATERIALS AND METHODS**

**Sampling**

During the present study, faecal samples (n=80) from diarrheic cases were used to evaluate the usefulness of the SYBR Green based real-time PCR assay for its diagnostic potential. The freshly voided stool samples were obtained from bovine calves (up to the age of 6 months) from different regions of India.

**Sample Preparation and Viral RNA Extraction**

Suspension of samples was prepared and stored as described in our earlier reports (Malik et al., 2014). Total RNA was extracted from 500 μL of the diarrheic faecal suspension using an equal volume of TriReagent-LS (Sigma-Aldrich, St. Louis, USA). The RNA was eluted finally with Nuclease Free Water (NFW) in a final volume of 25 μL. The isolated RNA was assessed qualitatively and quantitatively using Nanodrop Spectrophotometer (ND-1000, Thermoscientific, USA) and stored at -70°C until further use.

**RNA-PAGE and Primer Designing**

The genome of RV was detected and analyzed by RNA-PAGE following the method described earlier (Malik et al., 2011). The primers for NSP4 gene were designed by using GeneTore Lite 1.0 software (BioTools Inc., Edmonton, Canada) based on the highly conserved region from the sequence data available in the GenBank nucleotide database. The sense (nt position 458-5’TGGCGAATAGACAT-GAC 3’-475) and anti-sense (nt position 567-5’CGACGG-CACG TCAACCTTGA 3’-587) primers for 130 bp product length were selected and synthesized in the scale of 0.5 nmol by Metabion GmbH, Martinsried, Germany.

**Reverse-Transcription-Polymerase Chain Reaction**

Reverse-transcription for cDNA synthesis was carried out following procedures outlined in our earlier report (Malik et al., 2014). A PCR was carried out using the newly synthesized NSP4 primers with an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s, and extension at 70°C for 1 min, and a final incubation at 70°C for 10 min.

**Production of Plasmid**

The amplified product was run in agarose gel and NSP4 gene specific PCR product of 130 bp was excised from the gel using QIAquick Gel Extraction kit (Qiagen, Germany) and cloned into pJET1.2/blunt cloning vector (Fermentas, Lithuania). The clones were screened for the presence of the insert by Colony PCR and restriction digestion with 40U BglII (Fermentas, Lithuania). The positive plasmid was also confirmed through sequencing and sequence chromatogram was visualized in BioEdit v7.0.5 analysis software (Isis Therapeutics, CA, USA) and Mega Blast was performed with deduced sequences within the non-redundant nucleotide database (http://www.ncbi.nlm.nih.gov/BLAST) to confirm the presence of the NSP4 gene specific to RVA.

**Real-Time PCR**

Real-time PCR reaction conditions including annealing temperature were optimized using NSP4 plasmid construct. The optimum conditions with annealing temperature of 58°C, and primer concentration of 2.5 pMol/μl for the forward as well as the reverse primer gave amplification in all the positive samples with good ∆Rn values, highest sensitivity with negligible formation of primer dimers. The amplification efficiency and slope value of the real-time PCR assay were determined by amplifying duplicate amounts of the serially diluted plasmid DNA (10⁻⁷ to 10⁻⁹ copies) containing the 130 bp NSP4 gene insert. Real-time PCR was carried out in Strategene Mx3005 Real-Time PCR System (Stratagene, La Jolla, USA) using 1 μL of serially diluted plasmid DNA as a template, 10 μL of DyNAmoTM SYBR Green 2-Step qRT-PCR Master Mix (Finzymes, Finland) with ROX (0.3X) as passive reference dye. Cycling parameters for the real-time PCR consisted of initial denaturation for 10 min to activate the
Hot Start Taq DNA Polymerase, followed by 40 cycles at 95°C for 15s, 55°C for 30s and 72°C for 30s with the fluorescence data acquisition after the annealing and the extension steps. Melting curve analysis was performed for each sample to verify the specificity of each product which consisted of denaturation at 95°C for 1 min, annealing at 48°C for 30s and final denaturation at 95°C for 30s. The fluorescent data acquisition was continuous from annealing to the final denaturation step. The Real time data obtained were analyzed by MxProTM QPCR Software version 4.10 (Stratagene, USA).

**RESULTS AND DISCUSSION**

Real-time PCR assay with the optimized conditions was applied to screen clinical samples (n=80) obtained from diarrheic bovine calves with exception of that the template used was 2 μL cDNA instead of diluted plasmid standards. Melting curve analysis was performed for each sample to verify the specificity of the product(s). The samples whose melting curves shared the same T_m with the reference plasmid genotype standard and C_t values below 35 were interpreted as positive. Of the 80 faecal samples, 37 (46.25%) were detected positive with a C_t value of less than 35 and T_m value of 78.35°C ±1.0°C. RNA-PAGE analysis of the same samples showed a percentage positivity of 25% (20/80) with genomic migration pattern of 4:2:3:2 typical for RVA with ‘long’ electropherotype pattern of segments 10 and 11. Hitherto reports confirm use of RNA-PAGE for screening of RVs with percent positivity in the range of 3.3% (Jhala and Raghvan, 1997) in bovine calves from India. All the samples which showed segmented pattern in RNA-PAGE were detected positive in real-time PCR assay. Thus by using real-time PCR, the detection rate of RVs was in-
The higher detection sensitivity of the newly developed real-time PCR assay in comparison to RNA-PAGE is indicative of the suitability of this assay for screening of the field samples for bovine RVAs.

Though, to our knowledge, this is the first study in which a real-time PCR detection assay has been developed and applied for detection of bovine RVAs targeting more conserved genome segment 10, this newly developed SYBR Green based real-time PCR for NSP4 gene needs to be further validated with RV detection in other animal species with larger number of samples so as to better define its practical utility in molecular epidemiological studies and designing and adapting appropriate control measures. Detection of positive animals will be useful to monitor the RV infection within animal population and provide an early warning signal to predict an impending epidemic and risk for human population. In addition, the RVA specific real-time PCR developed in this study will be a useful tool for differential diagnosis of RV in a situation where a number of other agents like coronavirus, calicivirus, astrovirus etc. co-exist and are clinically indistinguishable.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, Indian Veterinary Research Institute for providing funds to carry out the present work. Special thanks to the staff and attendants from animal farms and hospitals who helped during collection of samples.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES


