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Research Article

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Development of VP6 Gene Specific Reverse Transcription (RT)-PCR Assay for Detection of Avian Group D Rotavirus in Diarrheic Chickens

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Abstract

The group D rotavirus (RVD) is an evolving viral cause of acute gastroenteritis in avian species world over. Despite the increase in the frequency of avian RVD infections world over, isolates from Indian poultry have yet to be characterised. Paucity of epidemiological studies with unavailability of sensitive and rapid detection methods for RVD in poultry led to design of the present study, aiming at development and validation of a RT-PCR assay, targeting the conserved group specific region of VP6 gene of RVD, for virus detection purpose. The detection limit of RT-PCR assay developed was superior ($2.95x10^{-7}$ ng/µL=$1.49×10^{3}$ copy numbers) to that reported previously ($5x10^{-4}$ ng/µL) from Brazil. The confirmation of the RT-PCR products was done through sequence analysis. The results confirm the diagnostic potential of RT-PCR assay for the epidemiological studies for RVD infection in growing poultry sector.

Keywords: Avian group-D rotavirus; RT-PCR; VP6 gene; Diagnosis; India

Introduction

Rotavirus (RV) is amongst the leading causes of acute gastroenteritis affecting several mammalian and avian species [1-3]. In poultry, it leads to colossal economic loss [4]. The virus belongs to the family *Reoviridae* and is a non-enveloped, 11 segmented, double stranded RNA genome virus [3]. The group specific antigenic determinants located within the inner capsid protein (VP6) classify the virus in to seven groups (A-G). Among these seven groups, rotavirus type A (RVA) strains predominates and affects both mammals and birds, while type D, F, and G are reported only in birds. For diagnosis, ribonucleic acid-polyacrylamide gel electrophoresis (RNA-PAGE) is an important technique which is more commonly used for confirmation of avian RV infection [4,5], but it suffers with the problem of sensitivity, making it inferior to other molecular detection methods available. Presence of avian RVs has been documented from India only on few occasions [6-8]. Though, RT-PCR based diagnostic assays have been employed for the detection for RVA, detection of group D rotaviruses (RVD) is still based on classical PAGE technique [8,9]. Paucity of epidemiological studies with unavailability of sensitive and rapid detection methods for RVD in poultry led to design of the present study. In this study, targeting group specific VP6 gene of avian RVD, a rapid RT-PCR assay for detection and confirmation of RVD infection in poultry was developed and validated.

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Materials and Methods

Group-D rotavirus samples

The RVD positive clinical sample obtained from a diarrheic enteritis case of chicken from Uttarakhand state of India was used for the development of RT-PCR assay. Suspension (10% w/v) of faecal sample in phosphate buffered saline (PBS) (pH 7.4; Sigma-Aldrich, St. Louis, USA) was centrifuged at 2000 g for 20 min to remove coarse particulate matter and the upper aqueous layer was filtered through 0.22 µm syringe filter and stored at -20°C until required.

Viral RNA extraction

The viral RNA was extracted from 500 µl of 10% faecal suspension in PBS using an equal volume of TriReagent-LS (Sigma-Aldrich, St. Louis, USA). RNA was eluted with Nuclease Free Water (NFW) in a final volume of 25 µl and assessed qualitatively and quantitatively using spectrophotometer (ND-1000, Thermo-Scientific, USA).

RNA-polyacrylamide gel electrophoresis (RNA-PAGE)

For the detection of 11 segmented dsRNA of rotavirus, the extracted RNA was subjected to RNA-PAGE as per the procedure described earlier [7,8]. For the electrophoresis, 10% resolving gel was used, using 12x10 cm plates and 12 well comb of AE-6450 gel slab casting apparatus (Atto, Corporation, Japan). The RNA samples (20 µl) were heated at 56°C for 5-10 min before loading into the wells. The gel was run at constant voltage of 70 V till the dye came out of the gel using 1X Tris glycine electrophoresis buffer. The gel was subjected to silver staining for visualization of the bands and the stained gel was photographed and stored in 10% ethanol.

Preparation of complementary DNA (cDNA) by Reverse Transcription (RT)

Reverse-transcription for cDNA synthesis from viral RNA was performed using 1.0 µL (100 ng/µl) random hexamer (Fermentas, Lithuania), 5 µL (100 ng) of viral RNA, and 2 µL of dimethyl sulphoxide (DMSO) added to PCR tube containing NFW, followed by incubation of the reaction mixture at 70°C for 5 min to melt secondary structures within the template. The mixture was immediately snap chilled on ice followed by the addition of 4 µL of 5X RT buffer, 2 µL of 10 mM dNTPs (Fermentas, Lithuania), 1 µL (40 U) RNase Inhibitor (Ambion, USA), 1 µL (200 U) MMLV-RT enzyme (Promega, USA) and NFW to make reaction volume of 25 µL and kept at 37°C for 60 min. The enzyme was denatured at 80°C for 3 min at the end of the incubation step to inactivate residual MMLV-RT. The cDNA thus obtained was used for PCR and the remaining was kept at –20°C till further use.

Designing of RVD specific primers

To design primers that can detect specifically RVD, 16 individual sequences of the VP6 gene of chicken RVD strains from different geographical locations were retrieved from the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore). The sequences were assembled using the ClustalW program of Lasergene 6.0 software (DNASTAR Inc, USA) for the alignment of all the retrieved sequences (Figure 2). Through VP6 gene nucleotide sequences alignment analysis, the conserved regions of VP6 gene of RVD (GenBank accession numbers of all RVD strains cited in Figure 2) were used for generating sense primer (position 579-600, 5'-GCTATACATTTCGCTGCATTTG-3') and anti-sense primer (position 764-743, 5'-TGGCCAATAGTGTGTGGCAGCT-3') using GeneTool Lite software (BioTools Inc., Edmonton, Canada) for a specific product size of 185 bp. All the primers were custom synthesized (Integrated DNA Technologies Inc., India).

Optimization of the RT-PCR assay

The plasmid construct of partial avian RVD VP6 gene (185 bp) was used as template for the optimization and evaluation of sensitivity of RT-PCR assay. Briefly, the RVD positive diarrheic faecal sample (UKD-34) was processed for viral ribonucleic acid (RNA) extraction using TriReagent-LS (Sigma, St. Louis, USA) followed by cDNA synthesis as described above. The partial length VP6 gene of avian RVD was amplified using sense and anti-sense primers (mentioned above). Various components of the RT-PCR reaction mixture included 2.5 µL of 10X PCR buffer mix, 0.5 µL of 25 mM $MgCl_2$, 0.5 µL of 10mM dNTPs, 1.0 µL each of forward and reverse primers (10 pmol/µL each), 2.0 µL cDNA, 0.25 µL of Taq polymerase (5 U/µL) and volume was make up to 25 µL with NFW. The PCR amplicon was visualized in ethidium bromide stained 1.5% agarose gel and documented using Transilluminator-UV®300 (UVP Inc., Upland, USA).

Cloning and sequencing

The specific RT-PCR amplicon (185 bp) was gel-purified by QIAquick Gel Extraction kit (Qiagen, Germany) and cloned into pGEM®-T Easy Cloning vector (Promega Corporation, USA). The clones were screened for the presence of insert by colony PCR and restriction digestion. The positive clones were further confirmed by sequencing using automated sequencer ABIPRISM®3100 (AME Bioscience, Toroed, Norway) at Scigenome Laboratories Pvt. Ltd. Kochin, Kerala. The plasmid was extracted from positive clones using GeneJET™ Plasmid Miniprep Kit (Fermentas, Lithuania) for further use as positive control and evaluation of sensitivity of RT-PCR assay.

Evaluation of the assay

For calculation of the sensitivity of developed RT-PCR assay, ten-fold dilutions (10^{-1} to 10^{-10}) of VP6 gene plasmid construct were used and PCR reaction was performed as mentioned earlier. The PCR amplified products were resolved in 1.5% agarose gel and visualized in Transilluminator-UV®300 (UVP Inc., Upland, USA). The specificity of the RVD VP6 gene primers was checked using RNA extracted from limited number of enteric viruses originating from different species viz. picobirnaviruses of bovine (GI/PBV/Bovine/India/HP/2012 strain H7, Acc. No. JX411966 PBV) and porcine origin (PBV/India/2013/NER/19P/ Acc.No.KJ650569), group A rotaviruses (RVA) of bovine (RVA/G6/India/UKD-PTN/2010/strain PBC, Acc. No. JX442784), porcine (RVA/E1/India/2013/strain C-3, Acc. No. KJ650568), avian (RVA/India/BE/2013/strain CH-22) and humans (RVA/G1/India/UK-HLD/2011/strain H180, Acc. No. JX411970). RNA from other enteric viruses were further confirmed with RVA and picobirnavirus specific primers. The assay was validated on 20 diarrheic poultry samples (one sample represents pool of one flock) collected from cases of diarrhea and enteritis in chickens in Northern parts of India during October 2012 to December 2013.

Results

RT-PCR assay optimization and evaluation

The optimum PCR conditions of initial denaturation at 95°C for 5 min followed by 35 cyclic denaturation at 94°C for 10 sec, annealing at 53°C for 30 sec and extension at 68°C for 30 sec with final extension at 68°C for 10 min yielded expected amplicon of 185 bp size. RNA from RVD positive sample and a negative control reaction mixture without template were analyzed using the above protocol and the PCR amplification was observed in RVD positive template with VP6 gene specific primers. *In vitro* analysis also confirmed that RVD primers were specific as none of sample from other enteric viruses (picobirnavirus, rotavirus group A from bovine, porcine, poultry and humans) yielded any positive amplification. Similar results were obtained during the *in silico* analysis of RVD primers with kobuvirus, bocavirus, coronavirus, parvovirus etc.

The sensitivity of RT-PCR assay for detection of RVD was found to be 1.49×10^3 copy numbers. The specificity of the primer pairs for RVD was analyzed using the proposed RT-PCR assay. The assay was also validated on 20 clinical samples obtained from the cases of diarrhea/enteritis in chicken, where 15% (3/20) samples were

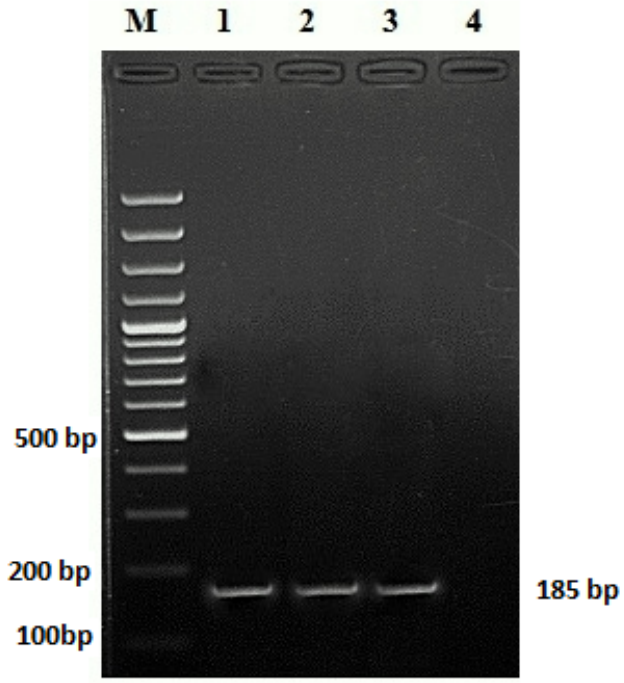

Figure 1: Agarose gel electrophoresis of VP6 gene based diagnostic RT-PCR products of avian group-D rotavirus. Lanes, M: 1 Kb Plus DNA Ladder (Thermo scientific); 1: UKD-119; 2: UKD-126; 3: UKD-142 and 4: NTC (Non template control).

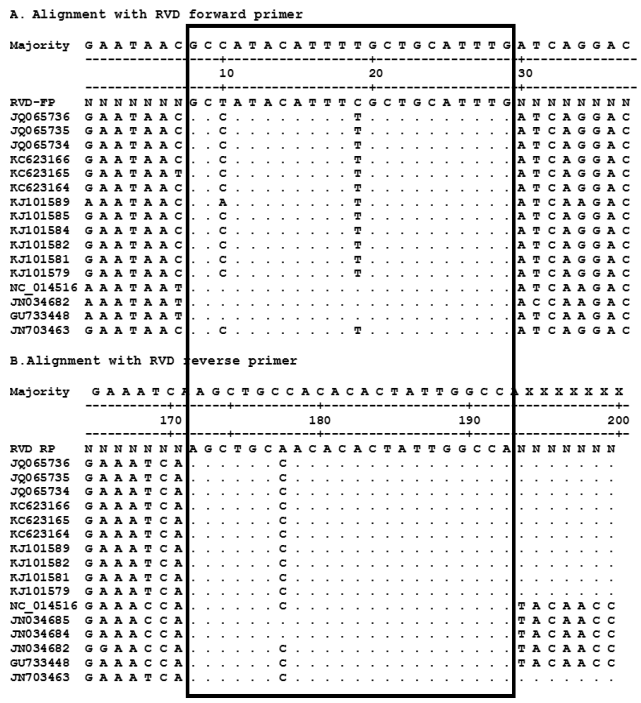

Figure 2: Sequence alignment of RVD primer regions. A. Alignment of forward primer and **B.** Alignment of reverse primer with other poultry RVD isolates. GenBank sequence accession numbers are provided.

detected positive for RVD with specific PCR amplicons (185 bp) in all the positive samples visualized in 1.5% ethidium bromide stained agarose gel (Figure 1). However, RNA-PAGE results confirmed group D specific migration pattern only in 2 samples out of 20 (10%). To testify the accuracy of the developed assay, all of the RT-PCR products from positive samples were sequenced which showed high sequence similarity with existing RVD isolates on BLAST analysis (data not shown).

Discussion

Rotavirus infection in poultry is of great concern throughout the world. The epidemiological reports confirm that new RV groups are evolving in poultry and might inflict the growing poultry sector in the coming years. The diversity and epidemiology of avian RVs throughout the world have been studied by several researchers [10-12], but epidemiological studies are barely accessible for other groups of RVs like D, F and G [13,14]. Currently, insufficient detection methodologies are available, predominantly for the new and emerging viruses. Amid the existing assays, RNA-PAGE is the one detection tool which is more frequently used for recognizing the avian RV infections throughout the world [4,9]. A timely identification of RV is, therefore, critical for control of disease and to minimize losses to the poultry sector. Of late, RT-PCR has been proved to be a powerful, sensitive and robust tool for RV detection, particularly for RVA infections [10], which is more predominant and affect a large number of mammalian species including human beings. Though, perusal of literature reveal that group D, F and G RVs have been recognized in avian species, RVD has only been confirmed in chicken [8-9,14-16].

In view of non-availability of nucleic acid based sensitive and specific techniques for easy and rapid detection of Indian avian RVD isolates, a RT-PCR assay was developed and evaluated. The primers designed targeting the conserved regions of VP6 gene of RVD and reaction mixture conditions were optimized. Though the region selected in VP6 gene is much conserved except few mismatches, the genomic configuration of RV makes it more prone for point mutations and genomic reassortment leading to emergence of newer strains. There is also every chance of accumulation of mutations in the primer region in VP6 gene of RVD isolates in days to come. To overcome the problem of primer mismatch, constant updation of primers would be required accommodating all variants of RVD. The sensitivity of the currently reported RT-PCR assay was found superior to the one reported by Bezerra et al. [17], in which detection limit was up to $5x10^{-4}$ ng/µL dilution, whereas in the current study detection was up to $2.95x10^{-7}$ ng/µL dilution which is equilent of $1.49x10^{3}$ copy numbers. Bezerra et al. [17] had not shown the limit in terms of copy numbers detected. In another study, Otto et al. [14] described detection of $2.65x10^{2}$ copies in the real-time PCR assay, however, such diagnostic assays are still expensive and are not affordable in developing countries. In comparison with conventional genome segment migration pattern based RNA-PAGE, in which 2 out of 20 samples were detected positive, the RT-PCR was found more sensitive where 3 of 20 samples showed specific amplification. The presently developed RT-PCR assay specifically detected the RVD isolates when checked with other limited enteric viruses.

After going through optimization, sensitivity and specific barrier checks, the RT-PCR assay was evaluated on a limited number (n=20) of clinical samples obtained from the cases of diarrhea/enteritis in poultry. The RVD isolates detected in the clinical samples by RT-PCR assay (Figure 1) were further confirmed by sequencing and NCBI blast analysis with other RVD isolates. The RVD positive samples from our previous study [18] based on 742 bp VP6 gene amplification for validation also yielded positive amplification using 185 bp primer set.

In conclusion, the results confirmed importance of VP6 gene specific RT-PCR assay developed in this study for detection of RVD in poultry as a rapid and more sensitive assay than the available in-use assays. This assay would enable precise detection of RVD infection in poultry and could therefore be invaluable for field diagnostic purposes. As the assay is rapid and reliable, most probably it has the potential to more widely integrate into routine molecular diagnostic techniques and in future could serve as an irreplaceable test highly useful in the epidemiological studies which will further strengthen the diagnostic tools for detection of RVD infection in growing poultry sector in India and the world. This appears to be the first report to develop RT-PCR based detection method for RVD from India.

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Conflict of Interest

There is no interest of conflict over the publication.

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