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RNA POLYMERASE GENE BASED RT-PCR ASSAY WITH PRIMERS UPDATE FOR GENUS SPECIFIC DETECTION OF PICOBIRNAVIRUSES

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ABSTRACT

Picobirnavirus (PBV) is an emerging causal agent of gastrointestinal and respiratory infections in humans and animals. Herein, a RNA polymerase gene based RT-PCR diagnostic assay was developed for initial detection of PBVs viral RNA in stool samples. Furthermore, sensitivity, specificity and validation of this assay were accomplished using field samples. The ORF2 (RdRp) coding region was chosen as the target, with more consensus portions within genus in the family *Picobirnaviridae*, tested in the genogroups I and II (GGI and GGII) of the virus. The RdRp nucleotide sequences from human and animal PBVs were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov) for primer designing. In this study, a total of 54 stool samples from animals (cattle calves (n=27), piglets (14) and goat kids (13)) suffering with diarrhea were screened. Expected size PCR amplicon of 275 bp was seen in 14 cattle calves, 12 piglets and 3 goat kids. The field validation of RT-PCR assay revealed presence of PBV viral RNA in 53.7% samples (29/54). The described genus specific assay could be applied for the primary detection of PBV infection in animals and, in combination with the genogroup specific PCRs, for the confirmation of new and emerging PBVs in animals.

Keywords: Picobirnavirus, RdRp gene, RT-PCR, New primers, Sensitivity, Specificity, Genogroups.

INTRODUCTION

The International Committee of Taxonomy of Viruses (ICTV) has proposed a complete new taxonomic order '*Diplornavirales'*, family *'Picobirnaviridae'* and genus '*Picobirnavirus*' (Malik *et al*., 2014a). Though members of *Picobirnavirus* (PBV) genus are known to cause enteric infections in mammalian (Bányai *et al*., 2003, Malik *et al*., 2011, Malik *et al*., 2014a, Bányai *et al*., 2014) and avian species (Silva *et al*., 2014), recent reports further add to their predilection for respiratory tract of humans and swine (Smits *et al*., 2011). Hitherto studies reports more common occurrence of PBVs in humans, poultry, pigs and bovine, though PBVs have also been identified in a number of other animal hosts such as camels, snake, monkey, sea lion etc., which could possibly rise the number of members in the genus or newer genus in the family (Fregolente *et al*., 2009). PBV is a simple structured naked, icosahedral symmetry virion with two double-stranded RNA genome segments (L and S). The larger segment 1 (2.2–2.7 kb) encodes the capsid protein, while smaller genome segment 2 (1.2–1.9 kb) encodes for the viral RNA-dependent RNA polymerase (RdRp) (Rosen *et al*., 2003, Wakuda *et al*., 2005, Duquerroy *et al*., 2009). The later segment exhibits extensive genetic diversity, and based on sequence configuration of RNA-dependent RNA polymerase (RdRp) gene (segment 2) PBVs have been classified into two genogroups i.e. Genogroup I (G-I) [Reference strain-1-CHN-97] and genogroup II (G-II) [Reference strain-4-GA-91] (Rosen *et al*., 2000, Bányai *et al*., 2003). Remarkably, more than 83% PBV sequences available in the National Center for Biotechnology Information (NCBI) belongs to genogroup I and less than 3% to genogroup II (Malik *et al*., 2014b).

Since, its first report in humans and black-footed pigmy rice rats in 1988 (Pereira *et al*., 1988), diagnosis of PBVs is quite challenging due to limited information on their genomic features as well as huge genetic diversity. The available detection methods include mainly RNA-PAGE and molecular (PCR) assays (Pereira *et al*., 1988, Rosen *et al*., 2000). RNA-PAGE has been used to separate two genomic segments by silver staining, but didn't yield reliable results and many a times due to lower sensitivity leaves PBV infections undetectable. The RT-PCR assay, initially employed for the detection of human PBVs by Rosen *et al*. (2000), is in use even for detection of animal PBVs. Availability of the PBVs sequences in gene sequence databanks over the time have shown vast variability in sequences of human and animal isolates and thus for the specific detection of members of the genus *Picobirnavirus*, with a broader detection range, an updated method for the detection of PBVs is necessitated. In this study, we directed RdRp gene consensus region with inclusion of degenerate sequences to cover mismatches. For evaluation of the herein developed specific RT-PCR assay, a range of PBV isolates from different animal species (cattle and pig) and human host were collected. The purpose of this study was to develop a broad host range specific RT-PCR assay for the specific and sensitive detection of PBVs.

MATERIALS AND METHODS

Virus isolates and total RNA extraction: For the development and validation of PBV genus-specific detection method, RdRp gene sequences from archived confirmed PBV isolates (based on sequence analysis) belonging to genogroups I and II from cattle (4 GG-I and one GG-II), pig (2 GG-I and 2 GG-II), goats (2 GG-I) and human (one GG-I) were used. In order to evaluate the specificity of the method, RNA isolated from rotavirus (RV) and astrovirus (AsTV) samples were included. Total RNA was extracted from either fresh or archived samples using TRIsoln (Merck, India) and RNA extraction method following manufacturer's instructions. All RNA extracts were kept at −80°C until used. Quantity and quality of the extracted RNA was verified by taking spectrophotometric OD at 260:280.

Primers designed for the PBV genus specific assay: RdRp gene coding sequence was preferred as the primers' target. RdRp gene has been the target of choice for development of nucleic acid based assays for a number of infectious agents (Dovas *et al*., 2003). For the primers design, 25 RdRp nucleotide sequences from human and animal PBVs were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov). The sequences were assembled and aligned using the Clustal W program of Lasergene 6.0 software (DNASTAR Inc, USA) (Fig. 1). Through RdRp gene nucleotide sequences alignment analysis, the conserved regions of PBV (GenBank accession numbers of all PBV strains cited in Fig.1) were used for generating primers. Several putative sites for primer design for the genus specific detection of PBVs were found after the alignment of the PBV RdRp sequences. Finally, from all the different primers tested, a primer set with sense primer PBV-7F (position 754-771, 5'-GCNTGGGTTAGCATGGA-3') and anti-sense primer PBV-7R (position 1028-1011, 5'-CAYGGNATGGSATSBGG-3') using GeneTool Lite software (BioTools Inc., Edmonton, Canada) for a specific product size of 275 bp taking Acc. No, AB517736 as reference, was selected for development of the generic detection method. All the primers were custom synthesized (Integrated DNA Technologies Inc., India). Primers melting temperature and the possibility of hairpins, self- and heterodimers formation, were calculated with OligoAnalyzer 3.1 (http://eu.idtdna.com/analyzer/Applications/ Oligo Analyzer/ Default. aspx–Integrated, DNA Technologies, US).

Complementary DNA (cDNA) preparation by Reverse Transcription (RT): A two-step RT-PCR was carried out for the specific detection of PBVs. The best combination of primers as well as the concentration of each reaction components, such as primers, template, enzymes, buffer system, and additives (DMSO) was assessed and optimized. Reverse-transcription for cDNA synthesis from viral RNA was performed using 1.0 µL (100 ng/µl) random hexamer (Fermentas, Lithuania), 10 µL (100-200 ng) of viral RNA, added to PCR tube containing 1 µL of nuclease free water (NFW), followed by incubation of the reaction mixture at 95°C for 5 min to melt secondary structures within the template. The mixture was immediately snap chilled on ice followed by the addition of 5 µL of 5X RT buffer, 1 µL of 10 mM dNTPs (Fermentas, Lithuania), 1 µL (40 U) RNase Inhibitor (Ambion, USA), 1 µL (200 U) MMLV-RT enzyme (Promega, USA) to make reaction volume of 20 µL and kept at 37°C for 60 min. The MMLV-RT enzyme was inactivated by keeping the reaction mix at 80°C for 5 min. The cDNA synthesized was used for PBV PCR using gene specific primers.

PBV genus-specific PCR: Genus-specific PCR for the detection of PBV isolates was carried out using 2 µl of cDNA, 1 µL of forward and reverse primers (10 pmole), 10 µL of 2X SapphireAmp Fast Master Mix (Takara Bio Inc., Japan) and 6 µL of NFW to make final volume of 20 µl reaction mixture. The optimum thermal cycling profile used was as: one first denaturation step at 95ºC for 5 min, and 40 cycles of (i) 94ºC for 20s, (ii) 48ºC for 45s, (iii) 72ºC for 45s, followed by a last extension step of 72ºC for 10 min. At the end of thermal cycling, amplicons were analyzed by electrophoresis in ethidium bromide stained 1.5% agarose gels and documented using in AlphaImager Mini (Cell Bioscience Inc., CA, USA).

Cloning and sequencing: The precise RT-PCR amplified product of 275 bp size was purified by using Gene-JET PCR purification kit (Thermoscientific, Lithunia). These eluted products were cloned in pGEM®-T Easy Cloning vector (Promega Corp., USA). The confirmation of the positive clones was made through colony PCR and restriction digestion. One of the positive clones was further confirmed by sequencing at Scigenome Laboratories Pvt. Ltd. Kochin, Kerala, India. The obtained sequences were analyzed using the BLAST algorithm. The plasmid was extracted from positive clone using GeneJET™ Plasmid Miniprep Kit (Thermoscientific, 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 the developed RT-PCR assay, ten-fold dilutions (10^{-1} to 10^{-10}) of RdRp plasmid DNA were used and PCR reaction was performed as mentioned earlier. The amplified products were resolved in 1.5% agarose gel and visualized in AlphaImager Mini (Cell Bioscience Inc., CA, USA). The specificity of the PBVs RdRp gene primers was checked using RNA extracted from other enteric viruses viz. rotavirus (RVA) and astrovirus. The RT-PCR assay developed herein was also validated on sequence confirmed PBV isolates from different animal

species (four GG-I and one GG-II from cattle; two GG-I and GG-II each from piglets; two GG-I from goats and one GG-I from human). Additionally, the developed PBV genus specific RT-PCR assay was used to screen 54 stool samples collected from different animal species including cattle calves (n=27), piglets (14) and goat kids (13) exhibiting clinical signs of diarrhea.

RESULTS AND DISCUSSION

Picobirnaviruses (PBVs) are amongst the emerging causes of gastrointestinal and respiratory infections in a wide range of animal species and humans, and thus are of major concern globally (Rosen *et al*., 2003, Bányai *et al*., 2014). Their presence, in immunocompromised patients, further intricate the situation. Hitherto studies provide limited epidemiological information on the PBVs due to non-availability of useful and practical diagnostic assays with field compliance. Presently, PBVs diagnostic methodologies include polyacrylamide gel electrophoresis (PAGE) and reverse-transcription-PCR, where former one suffers with lower sensitivity, leaving PBV infections undetectable on several occasions, while later one may agonizes as PBVs from different animal species and human origin display enormous genetic diversity. This makes difficult to select a consensus portion for primer design for RT-PCR assays, seeking need for primer updating with more frequency of degeneracy to cover emerging virus population. Till now, human-PBV primers proposed by Rosen *et al*. (2000) are in use to detect human and animal PBVs. Lately, metagenomic approach has helped in elucidating the etiology of diarrheal disease in several mammalian and avian species, providing evidence on the diversity of the animal faecal virome and addition of PBV with new host species (Fregolente *et al*., 2009). PBVs have been detected in dromedary's camels and bats (Woo *et al*., 2014). Nevertheless, molecular characterization of a few PBV isolates, has led to advances in our understanding of the virus, however the use of highly accurate, reliable and sensitive approaches to diagnose PBVs is still in its infancy and for generating epidemiological information on the PBVs there is an exigent need of a practical diagnostic assays with field compliance.

The prolonged infection and faecal shedding has allowed understanding into the intra-host evolution of PBVs, which have distinctly specified significant divergence of genomic sequences over a relatively short period (Masachessi *et al*., 2012, Masachessi *et al*., 2015). Furthermore, in addition to accumulation of point mutations that leads to significant sequence diversity in PBVs, recent findings provide evidence of reassortment events within PBV genomes (Bányai *et al*., 2014). Sequence analysis studies suggest huge sequence diversity and varying evolutionary dynamics of PBVs in different host species, thus hindering in development of a sequence based nucleodiagnostics. One of the most fundamental steps in the development of such a generic method is the selection of the primers annealing site and their design. The RNA polymerase (RdRp) coding sequences have been preferred as the primers' target, due to their highly conserved motifs between different species or even genus, for developing generic detection methods (Dovas *et al*., 2003, Maliogka *et al*., 2004).

In the present study, a two-step RT-PCR assay was developed, capable of detecting the genus PBVs in animals and human. Different primers and cycling protocols were tested for the optimization of the genus specific PCR targeting RdRp coding sequences of genome segment 2 (ORF2). For the RT-PCR of the PBV viral RNA, the primer that gave the best results, in terms of specificity and sensitivity, was PBV-7F and PBV-7R (Table 1). These diagnostic primers were planned to have analogous Tm, so as a single thermal cycling profile can be used for all the reactions. The expected Tms for the forward and reverse primers varied from 53.6 °C to 55.3 °C PBV-7F is a degenerate primer and has a single degeneracy at its third nucleotide position from the 5'end, whereas PBV-7R has penta degeneracy at its respective positions shown in Fig. 1, which is used for covering the broad PBV host range, including different animal species PBVs. For calculation of the sensitivity of the developed RT-PCR assay, ten-fold dilutions (10^{-1} to 10^{-10}) of RdRp plasmid DNA, were used and PCR reactions were performed, as mentioned earlier. The detection limit of RT-PCR assay developed was $7.9x10^{1}$ copy numbers (Fig. 2) with RdRp plasmid DNA. The detection limit of Rosen *et al*. (2000) primers set is not available, thus could not be interrelated. Higher degeneracy in reverse primer of PBV (PBV-B43) was also proposed by Rosen *et al*. (2000), where four degenerate primers were used for genogroup I detection. In the event of evolution of PBV strains with huge genetic diversity, the primer pairs proposed by Rosen *et al*. (2000) for genogrouping of GGI and GGII showed blocking at 3'end of many of the PBV isolates. The newly designed primers are genus specific and useful for initial detection of PBV followed by genotyping of the isolates using previously published primers.

The RT-PCR assay that was developed for the genus-specific detection of the PBVs produced the expected 275 bp (base pairs) amplicons (Fig. 2). Its detection range was broad as the developed method was capable of detecting PBV isolates from different host species including pig, goats, cattle and human. Unfortunately, we were not able to obtain an adequate number of isolates from other farm animal species such as equine, camels and thus it was not possible to assess the primers' detection range for PBVs for these species. The RT-PCR developed herein was capable of detecting all the sequence confirmed GG-I and GG-II PBV isolates

Table 1. Primers used during the study for detection and geno grouping of picobirna viruses.

Gene/ PBV Genogroup	Primer sequence 5'……3'	Position*	Size (bp)	References
	Oligoprimers used to detect picobirnavirus			
RdRp gene amplification	Forward- PBV-7F, 17 bp 5'- GCNTGGGTTAGCATGGA -3'	754-771	275 bp	Reference strain/ sequence
	Reverse-PBV-7R, 17 bp 5'- CAYGGNATGGSATSBGG -3'	1028-1011		GPBV10 (AB517736) This study)
	Oligoprimers used for genogroup picobirnavirus (GG-1 and GG-II)			
GG-1 gene amplification	Forward- PBV-B25-GG1-F, 15 bp 5'-TGGTGTGGATGTTYC-3'	665-679	201 bp	Reference strain/ sequence
	Reverse-PBV-B43-GG1-R, 16 bp 5'-ARTGYTGRTCGAAYTT-3'	865-850		1-CHN-97/China (AF246939) Rosen *et al*. (2000)
GG-II gene amplification	Forward- PBV-B23-GG2-F, 15 bp 5'-CGGTATGGATGTTTC-3'	685-699	369 bp	Reference strain/ sequence
	Reverse- PBV-B24-GG2-R, 15 bp 5'-AAGCGAGCCCATGTA -3'	1053-1039		4-GA-91/USA (AF246940) Rosen *et al*. (2000)

* Position as per the RdRp gene of reference strain/sequence given.

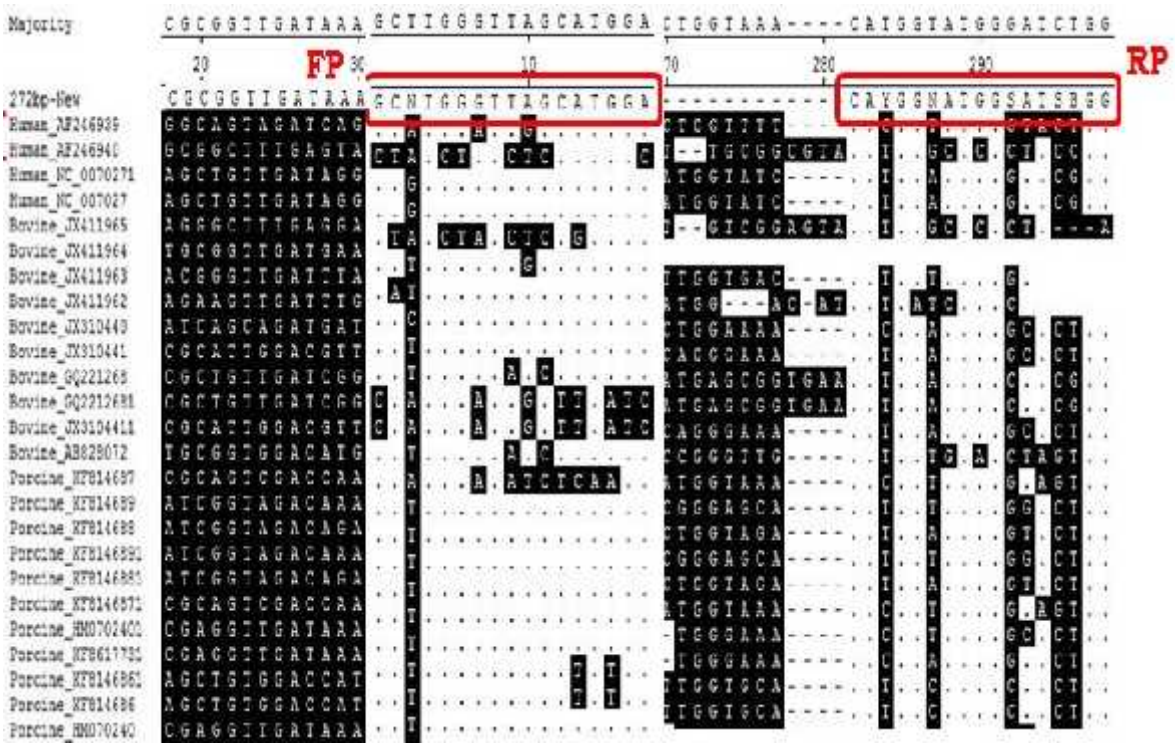

Figure 1: Alignment report of newly designed 275 bp primers. PBV sequences retrieved from NCBI GenBank are provided with species of origin and accession numbers. Dots show similarity at nucleotides. Degeneracy indicates N=A/T/G/C, Y=T/C, S=C/G and B=C/T. FP-Forward primers; RP- Reverse primer

from animals and human (Four GG-I and one GG-II from cattle, 2 GG-I and 2 GG-II from piglets, 2 GG-I from goats and one GG-I from human). The method also proved to be highly specific as no amplicon was generated from the RNA obtained from rotavirus and astrovirus isolates. Additionally, screening of the 54 field samples [cattle calves (27), piglets (14), goat kids (13)] by the developed RT-PCR revealed a total of 29 (53.77%) samples positive (14/27 cattle, 12/14 piglets and 3/13 kids). Of these 29 PBV positive samples, 24 (82.75%) could further be genotyped as GG-I (n=21) and GG-II (n=3) with published primers of Rosen *et al*.

(2000). The failure in genotyping of the 5 samples could be due to sequence mismatch with the published genotyping primers, as reported in several other highly diverse viruses (Malik *et al*., 2013). Hitherto studies have revealed high sequence diversity among PBVs (Bányai *et al*., 2014), however sequencing of 275 bp amplicon from these 5 positive samples, which could not be genotyped, confirm their sequence homology with PBV genogroup I. Thus, it appears that Rosen *et al*. (2000) primers might have produced false negative results. Therefore, primer updating to accommodate emerging PBV isolates is further necessitated. Sequencing and Blast analysis of selected generic PCR amplicons (275 bp) generated from PBV isolates collected from cattle, goats, pig and humans showed their PBV genus origin. PBV isolates from cattle, pig and human showed amino acid sequence similarities of less than 60% with PBV virus isolate sequences deposited in the databases (data not shown).

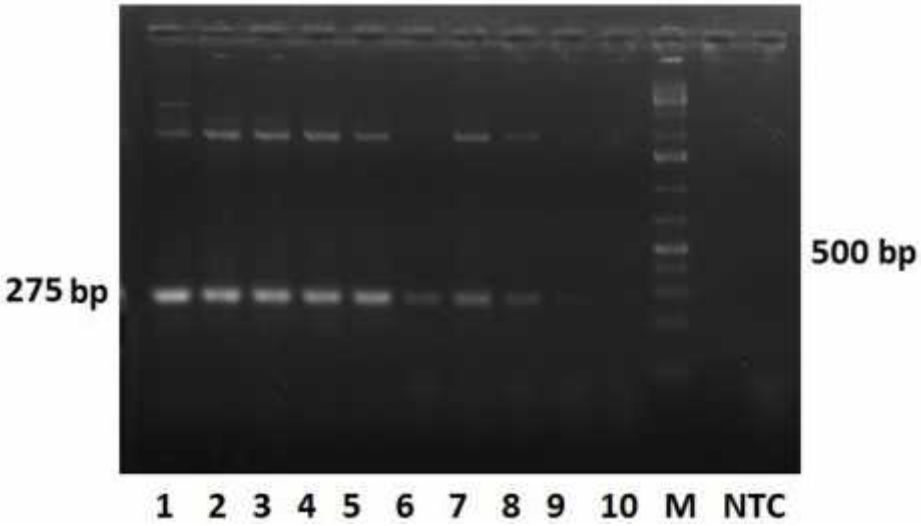

Figure 2: PCR with new PBV primer set (PBV-7F and PBV-7R) with ten-fold dilutions (10^{-1} to 10^{-10}) of plasmid DNA. After PCR thermal cycling, dilutions from 10^{-1} (lane 1) to 10^{-10} were loaded (lane 10) on 1.5% agarose gel stained with ethidium bromide. Specific amplicon of 275 bp sizes were seen from lane 1 to 9. No amplicon was seen in non-template control (NTC). Lane M indicates 1kp plus ladder (Fermentas, Lithuania).

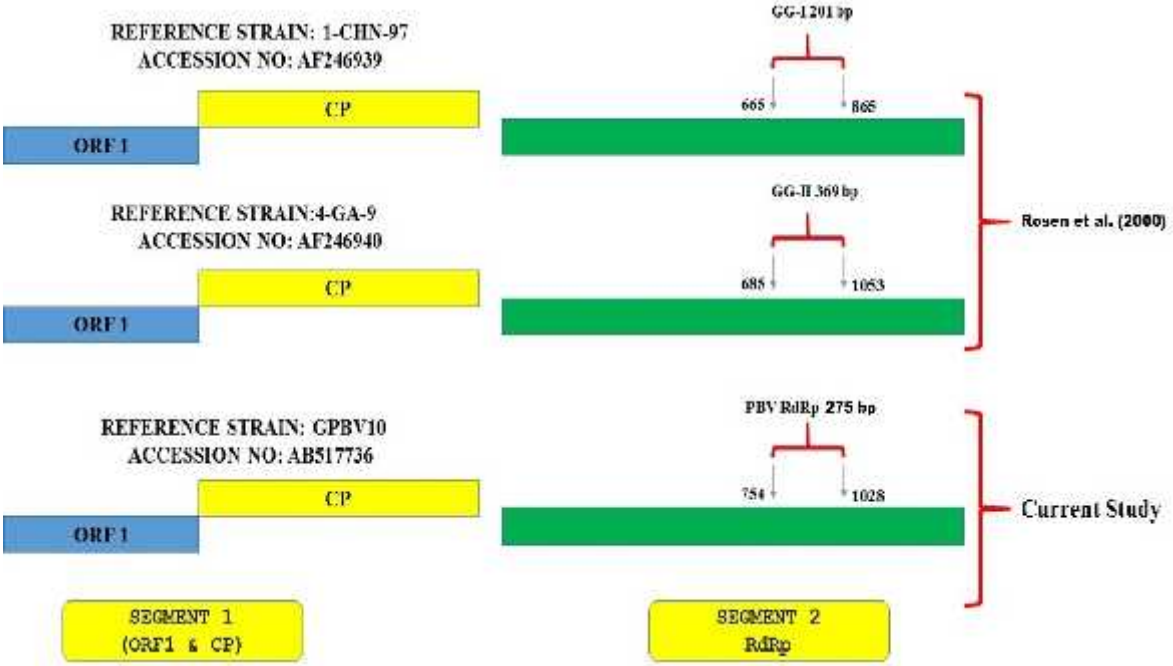

Figure 3: Schematic representation of position of genogrouping and in-house designed primers within the reference PBV sequences.

Conclusions: Overall, the developed assay could amplify efficiently the corresponding 275 bp product of the RdRp gene from all the viral isolates tested, originating from different host species, without any non-specific detection outside PBVs. The *Picobirnaviridae* family is still growing and new susceptible host species are being added regularly and naturally the accumulation of more data will increase our understanding of PBVs. Therefore, the methods developed herein will contribute toward this direction by providing a reliable and sensitive detection of PBVs and would further help to review disease burden and envisage new instructions for accepting progress in public health actions. Recent studies have highlighted persistence of PBV infection in asymptomatic carriers, where PBV infection acquired during early stages of life could maintain until the beginning of adulthood confirming that the PBV-host interaction proves as a persistent and asymptomatic infection. The close relatedness of animal PBVs with human along with detection of PBVs from the sewage designate the potential threat in terms of infection acquirement from the sewage and transmission of these viruses across the species. Thus, the new genus specific RT-PCR assay with updated primer sets covering major animal PBV isolates could find field compliance and would be useful in detecting PBVs in early stages of infection and asymptomatic co-existence also.

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