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Occurrence of Neglected Picobirna Virus in Buffalo Calves Associated with Diarrhoea

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Abstract

Picobirna viruses (PBVs) thought to be neglected viruses, are emerging as causative agents of viral gastrointestinal infections in human and other animal species with enteritis. In the present study, a total of 408 fecal samples of diarrheic buffalo calves of under age of 2 months were collected from different locations in Haryana. These samples were preliminarily screened for PBV by RNA-polyacrylamide gel electrophoresis (RNA-PAGE). The RNA-PAGE positive samples were further confirmed by RT-PCR using RdRp gene specific primer pair PicoB25 (+) and PicoB43 (-) for amplification of genogroup I PBV that generated an amplicon of 201 bp. Out of 408 buffalo calves fecal samples, 52 (13%) samples were found positive for genogroup 1(GGI) PBV using RT-PCR. Genogroup II (GGII) specific primer generating 369 bp product size did not show amplification in any of the samples. The results suggested occurrence of PBV in Haryana State with predominance of GGI PBV. To the best of our knowledge, this is the first report of detection as well as occurrence of GGI picobirna virus among diarrheic buffalo calves in Haryana State.

Key words: Buffalo calves, Bisegmented dsRNA genome, Genogroup I PBV, RT-PCR, Viral diarrhea.

Introduction

The picobirna virus (PBV) is a small, non-enveloped (33-35 nm) bisegmented novel double stranded RNA (dsRNA) virus belongs to order *diplornavirales* under family *picobirnaviridae* (Fregolente *et al.,* 2009; Knowles *et al.,* 2012; Tapparel *et al.,* 2013). PBVs were first time identified in free-living *Oryzomys nigripes* rats by PAGE using ethidium bromide or silver nitrate staining which showed two sharply defined bands (Pereira *et al.,* 1988). PBVs are excreted in stool samples of broad range of hosts. However, information regarding their genomes is limited to viruses detected from porcine, rabbits and human species only. They were classified as opportunistic diarrhoeagenic pathogens, rather than potent pathogens, associated with viral gastroenteritis. The genome size of PBV ranges from 2.36 to 2.69 kbp for larger and 1.58 to 1.7 kbp for smaller segment (Costa *et al.,* 2004; Malik *et al.,* 2014). The name picobirna virus was proposed based on its small size genome and two segment genomic pattern. Because of its unique genomic and morphological characteristics, this novel group of viruses is a potent candidate to place in a new taxon (Knowles *et al.,* 2012). The larger RNA segment (segment 1) possesses two open reading frames (ORF) which encodes amino acid fragments having 224 and 552 amino acid residues. However, small RNA segment (segment 2) consists of a single open reading frame and encodes amino acid fragment of 534 amino acid residues. The small segment encodes amino acid motifs typical of those encoded by the RNA-

dependent RNA polymerase (RdRp) genes. The genogroup based RT-PCR using specific primer against RdRp gene (segment 2) grouped PBVs into 2 genogroups, i.e., genogroup I (GGI) and genogroup II (GGII) (Bhattacharya *et al.,* 2007). However, the epidemiology of PBVs has not been well understood till date and in this regard only scanty information is available in the literature. The scanty sequencing data of PBVs of faecal samples of different animal species provided only limited information to investigators to establish whether PBV circulation is influenced by host species restriction. The aim of this study was to investigate the frequency of occurrence of PBV infection in dairy buffalo calves and to perform the molecular characterization of buffalo PBV strain detected in faecal samples from diarrhoeic calves.

Materials and Methods

Sample collection and viral nucleic acid extraction

A total of 408 fecal samples were collected from buffalo calves under the age of 2 months from different locations of Hisar (local organized farms, College of Veterinary Sciences, postmortem hall and veterinary clinics, LUVAS). All the fecal samples were collected from diarrhoeic animals in screw capped plastic vials. Samples were sent to lab on ice. All the collected fecal samples were suspended in PBS buffer to make a 10% suspension. Total viral RNA was extracted by GIT lysis method as described by Minakshi *et al.* (2005). Extracted viral RNA was stored at -20°C till further use. The segmented viral RNA genome was analyzed on 8% RNA-polyacrylamide gel electrophoresis for two hours at constant voltage followed by silver staining of the gel to visualize the RNA segments as described earlier (Svensson et al., 1986; Minakshi *et al.,* 2005).

cDNA synthesis and amplification by polymerase chain reaction (PCR)

Viral genomic RNAs of RNA-PAGE positive fecal samples were isolated by TRIZOL® (TAKARA, Japan) method following manufacturer's instructions and were used for cDNA synthesis using random primers and moloney murine leukemia virus-reverse transcriptase (Mo-MuLV-RT) enzyme (Promega, USA). Reverse transcription reaction was carried out in a 20 µl reaction mixture using 1.0 µL (100 ng/µl) random hexamer (Fermentas, Lithuania), 1.0 µL of DMSO, 3µL (200-500 ng) of viral RNA, and 3µL of nuclease free water (NFW). RNA and primer mixture was heated at 99°C for 5 min in thermal cycler (BIORAD, USA) to melt secondary structures within template. The mixture was immediately snap chilled on ice followed by addition of 12 µl of reaction mixture {1.0 µl (200 units/µl) of Mo-MuLV-RT, 2.0 µl of10X RT buffer, 1 µl of 10 mM dNTPs and 8.0 µl of nuclease free water (NFW)} to make reaction volume 20 µl. After allowing the primers to anneal at 25°C for 10 min, reverse transcription reaction was carried out at 37oC for 60 min in thermal cycler. The reverse transcriptase was heat inactivated at 70°C for 10 minutes. The cDNA was stored at -20°C till further use.

The cDNAs were amplified by PCR using PBV group specific primers. Two different sets of primer pairs were used to amplify the 201 bp (Banyai *et al.,* 2003) and 369 bp (Rosen *et al.,* 2000) fragment of the RNA dependent RNA polymerase (RdRp) gene of picobirna virus 1(PBVI) and picobirna virus II (PBVII), respectively (Table 1). The reaction conditions for PCR were standardized to get the desired gene specific PCR products. PCR reaction was standardized in 20 µl reaction volume. An

Table 1: List of primers used to amplify targeted sequences of Picobirna virus

Genotype	Primer name	Sequence (5'-3')	Product size (bp)
PicobirnaGpI	PicoB25F	TGG TGT GGA TGT TTC	201
	PicoB43R	AGT GGT CGA ACTT	
PicobirnaGpII	PicoB23F	CGGTATGGATGTTTC	369
	PicoB24R	AAGCGAGCCCATGTA	

aliquot of 10 µl of 2X Master Mix (TopTaq® Master Mix Kit, Qiagen), containing forward and reverse primer 0.5 µl (1 mM) each, nuclease free water (7µl) and cDNA (2 µl). The amplification was carried out in a thermal-cycler with cyclic conditions specific to genogroup specific primer. The initial denaturation step was kept at 94°C for 5 min followed by cyclic denaturation at 94°C for 30 sec and extension at 72°C for 30 sec. The final extension was kept at 72°C for 7 min for both set of primers. Annealing temperature was kept 57°C for 50 sec for GGI and 57°C for 60 sec for GGII for each primer pair depending on their melting temperatures. The numbers of PCR cycle were kept 30 for both set of primers. The RT-PCR products were analyzed on 2% agarose gel electrophoresis in 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA), pH 8.4, stained with ethidium bromide (0.5 ng/mL) and visualized under UV transilluminator (GeNei™ UVITEC Imaging Systems, Cambridge).

Results and Discussion

Out of 408 buffalo calves' diarrhoeic faecal samples, 52 (13%) samples were found positive for picobirna virus infection by both RNA-PAGE (Fig. 1) and RT-PCR assay using RdRp gene specific primers, whereas Takiuchi *et al.* (2016) reported 8.3 % prevalence only. High incidence of PBVs in Haryana state suggests their association with diarrhoea. All the positive samples showed PCR amplicon of 201 bp using RdRp gene specific primer pair (PicoB25 and PicoB43) which is specific for picobirna virus genogroup 1 (Fig. 2). None of the sample was detected positive for genogroup II virus.

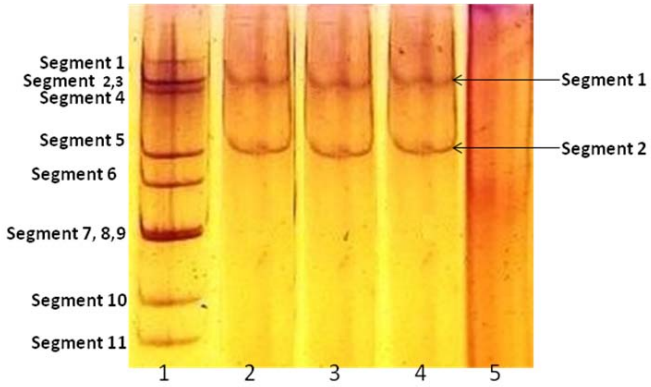

Fig. 1: Migration pattern of genomic dsRNA segments of different picobirna virus isolates in RNA-PAGE.Lane 1: buffalo rota virus (reference strain); Lane 2-4: Buffalo picobirna virus; lane 5: negative faecal sample

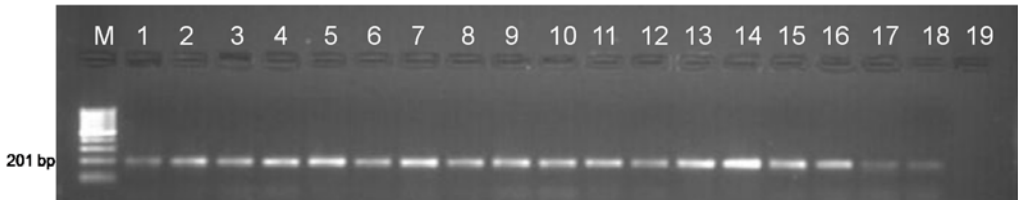

Fig. 2: Amplification of buffalo Picobirna virus genotype I RdRp gene by RT-PCR using RdRp gene specific primer pair. Lanes: M: 100bp Ladder; 1: Positive control for picobirna virus; 2-18: Positive Field Sample of Buffalo PBV; 19: NFW Negative control.

In the present study RNA-PAGE has been found suitable and reliable method for PBV detection as this method provides sufficient resolution to fractionate the two viral RNA segments from other nucleic acids in fecal samples and can also distinguish the electropherotypic profiles of PBVs as observed by other workers (Rosen *et al.,* 2000; Buzinaro *et al.,* 2003). Twenty years ago, in our laboratory, buffalo PBVs were detected in a few cases during buffalo rota virus research in diarrheic and non-diarrheic faecal samples with two genomic segments; but they were ignored as negligible/ neglected viruses due to not having any role in calf diarrhoea. Although the prevalence of buffalo PBV are still scanty in the literature but at the same time prevalence of 13% of buffalo PBV in our

study was much higher than those reported in rest of studies. Several epidemiological studies involving human and animal PBVs detection, only few reports described the presence of PBV in feces of cattle or buffaloes in Belgium (Vanopdenbosch and Wellemans, 1989), in Brazil (Buzinaro *et al.,* 2003) and in India (Ghosh *et al.,* 2009; Malik *et al.,* 2011, 2013, 2014). Picobirna virus has also been detected in Japanese cattle fecal samples by RT-PCR (Nagai *et al.,* 2015). Bhattacharya *et al.* (2007) observed large genomic profile of picobirna virus with two genomic segments of 2.3 to 2.6 kb (segment 1) and 1.5 to 1.9 kb (segment 2) with 2.47 % prevalence of small genome profile of genogroup I picobirna virus. Large genome profile of picobirna virus among diarrhoeic and non-diarrheic human cases with prevalence rate of approximately 0.45% was reported by Pereira *et al.* (1988). Buffalo genogroup I PBV was reported in Bengal (Ghosh *et al*., 2009) with subsequent detection of genogroup 1 picobirna virus in fecal samples of diarrheic bovine calves from same area (Mondal and Joardar, 2014). Prevalence of large genome profile of PBV in faecal samples of diarrhoeic cattle and buffalo calves has also been reported from western India (Mondal *et al.,* 2013), without any detection from Haryana state in northern India. It has been reported that at molecular level buffalo genogroup 1 strains characteristics of bubaline PBV in Maharashtra are different from that of human and other mammalian hosts (Malik *et al.,* 2013).

Conclusion

The current study was conducted with the aim to find out occurrence of PBV infection in buffalo calves from Haryana state, India. All the RNA-PAGE positive PBV samples (13% of 408) showed PBV group I specific amplicon of 201 bp only. None of the samples showed PBV group II specific (369 bp) amplicon. Thus, predominant infection of genogroup I PBV was found in buffalo calves in Haryana. The result confirmed the epidemiological evidence of presence of genogroup I PBV in diarrheic calves. To the best of our knowledge, this is the first report of PBV identification in diarrheic faecal samples of buffalo calves from Haryana. However, molecular tool based further survey on large number of samples from different hosts species is required to reach at a better conclusion regarding emergence and pathogenesis of PBV.

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Conflict of interest: Authors declare no conflict of interests that could possibly arise.

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