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Porcine Astrovirus Detection and Characterization in Healthy and Diarrheic Pigs from Haryana, India

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ABSTRACT

Background: Porcine astrovirus (PAstV) is an emerging pathogen detected from the feces of both healthy and diarrheic pigs with limited studies comparing the presence of PAstV and diarrhea in pigs. PAstV has been reported worldwide with a few reports from India. In addition, there are limited reports on variation in the amino acid sequence of the ORF2 region, which codes for viral capsid protein. The present study was aimed to study the presence of PAstV, association with diarrhea in pigs of various age groups and its molecular characterization from Haryana, a northern Indian state.

Methods: A total of 176 rectal swabs of pigs were collected from different parts of Haryana. The samples were screened for PAstV by RT-PCR of the partial ORF1b/ORF2 genomic region. The positive samples were characterized by molecular typing and phylogenetic analysis.

Result: In the present study, PAstV was detected in pigs in 16.47% of fecal samples, including 21/127 (16.53%) diarrheic and 8/49 (16.32%) non-diarrheic. The results indicated that weaning piglets were more susceptible to PAstV infection followed by suckling piglets. The phylogenetic analysis revealed the circulation of PAstV4 and PAstV2 lineages in Haryana. The study revealed endemic presence of virus in the region with high genetic diversity among the PAstV strains, suggesting a wide range of heterogeneity.

Key words: Molecular detection, ORF 1b/ORF2, Porcine astrovirus.

Abbreviations: AstV: Astrovirus, ORF: Open reading frame, PAstV: Porcine astrovirus, RdRp: RNA dependent RNA polymerase.

INTRODUCTION

Porcine astrovirus (PAstV) belongs to the family *Astroviridae*, which comprises of two genera, *Mamastrovirus* and *Avastrovirus,* isolated from mammals and birds, respectively. The PAstV virus is a spherical, non-enveloped virus with a distinct star-like morphology and a size of about 28-30 nm. The virus contains a single-stranded positive-sense RNA with genomic length of about 6.4-7.3 kb (ICTV). The genome is divided into three open reading frames (ORFs) termed as ORF1a, ORF1b and ORF2 flanked by untranslated regions (UTRs) at both the 5′ and 3′ ends of the genome. The non-structural proteins required for viral replication and transcription include serine protease, viral genome linked protein, transmembrane helices encoded by ORF1a and RNA dependent RNA polymerase (RdRp) encoded by ORF2 gene (Zhao *et al*., 2019). The PAstVs have been further classified into five distinct lineages (PAstV1-5) based on the phylogenetic analysis of the ORF1b and ORF2 sequences (Lee *et al*., 2013; Qin *et al*., 2019).

The PAstV has been reported in diarrheic (Moser *et al*., 2005) as well as non-diarrheic pigs (Kumthip *et al*., 2018; Qin *et al*., 2019); however, its association with diarrhea is not well understood (Cai *et al*., 2016; Rawal and Linhares, 2022). In addition, PAstV has also been associated with respiratory infections and more recently with polyencep halomyelitis, indicating its greater clinical importance (Boros *et al*., 2017; Padmanabhan *et al*., 2016; Xiao *et al*., 2013). PAstVs is reported to be endemic in several European, North and South American countries and less common in Asian countries (Flores *et al*., 2021; Kumthip *et al*., 2018). However, very few researchers have examined the relationship between gastrointestinal disease and PAstV globally (Fang *et al*., 2019; Kumthip *et al*., 2018; Rawal and Linhares, 2022).

There are two confirmed reports of the occurrence of PAstV in diarrheic pigs from India, 17.6% from Assam, Meghalaya, Mizoram, Tripura, Nagaland, Manipur, Karnataka, Tamil Nadu, Kerala and Uttar Pradesh (Kattoor *et al*., 2019) and 31.8% from Haryana (Kaur *et al*., 2021). However, no study has been carried out for the detection of

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PAstV from non-diarrheic pigs in India. In addition, there are limited reports on variation in the amino acid sequence of the ORF2 region, which possesses neutralizing epitopes and sites for viral attachment to the host cells. The present study was therefore conducted with the aim to study the presence of PAstV, association with diarrhea in pigs of various age groups and its molecular characterization from Haryana, a northern Indian state.

MATERIALS AND METHODS

The study was conducted at the Department of Veterinary Microbiology, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, India. A total of 176 rectal swabs were collected randomly from 14 piggery units located across different parts of Haryana during September, 2019 to February, 2020 (Fig 1). The samples were collected from both diarrheic (n=127) and in-contact clinically healthy (n=49) pigs of different age groups. A total of 33 samples were collected from suckling pigs (0-3 weeks), 87 samples from weaning pigs (3-9 weeks), 37 samples from fattening pigs (9-24 weeks) and 19 samples from adult pigs (>24 weeks) (Table 1). The collected samples were stored at -20°C till further processing.

The RNA was isolated using Trizol LS (ThermoScientific, USA) with 200 µL of fecal suspension as the starting volume and the final RNA was dissolved in 25 µL of nuclease free water as per the manufacturer's instructions. The reverse transcription was performed using random hexamers with Revert aid first strand cDNA synthesis kit (Thermo Scientific, USA) as per the manufacturer's protocol. The PCR was performed using a primer pair for the partial ORF1b/ORF2 region, PAstV-F (5′-TGACATTTTGTGGATTTACAGTT-3′) and a reverse primer, PAstV-R (5′-CACCCAGGGCTGACCA-3′) (Reuter *et al*., 2011; Kumthip *et al*., 2018). The PCR was carried out using 10 µL of 2× Dream Taq green PCR master mix (Thermo Scientific, U.S.A.), 0.6 µL each of 10 µl forward and reverse primer, 2 µL cDNA and nuclease-free water to make up to 20 µL of the total reaction volume. Amplification conditions were optimized in the ProFlex PCR System (Thermo Scientific, USA) at initial denaturation of 95°C for 4 min, followed by 35 cycles of denaturation temperature at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 7 min. The PCR products were validated by electrophoresis in 1.5% agarose gel and visualized with ethidium bromide using the gel documentation system (Azure Biosystem, U.S.A.).

The PCR products with an expected size of 799 bp were gel purified using the QIAquick gel extraction kit (Qiagen, Germany). The purified products were sequenced commercially by Genebio Solutions, Dehradun, India in both directions using Sanger's sequencing method. These nucleotide sequences were phylogenetically compared with sequences of human AstV, rodent AstV, bovine AstV, deer AstV and other PAstVs retrieved from the NCBI database. The alignment was done using the Clustal W program in MEGA X software and the phylogenetic tree was constructed using the maximum likelihood method and the Tamura-Nei model with gamma distribution, keeping the bootstrap value at 1000 replicates (Kumar *et al*., 2018). Further, the deduced amino acid sequences of the partial ORF2 region were used for the construction of the phylogenetic tree using the maximum likelihood method and the Jones-Taylor-Thornton (JTT) model with gamma distribution, keeping the bootstrap value at 1000 replicates (Kumar *et al*., 2018).

RESULTS AND DISCUSSION

In the present study, 16.47% (29/176) rectal swab samples from pigs were tested positive for PAstV by RT-PCR of partial ORF1b/ORF2 region (Fig 2). Our findings were consistent with previous studies that found 17.6% of PAstV in India

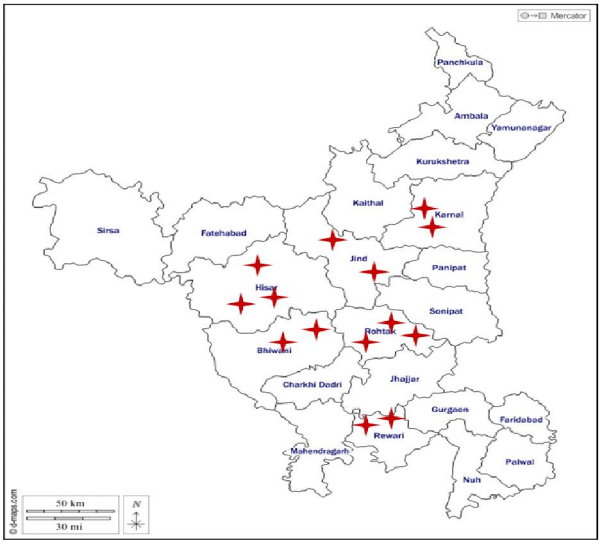


Fig 1: Map of Haryana showing the districts from where pig rectal swab samples were collected during the period September 2019 to February 2020.

Table 1: Detection of PAstV by RT-PCR in pigs of Haryana, India.

Age categories	Diarrheic pigs	Non-diarrheic pigs	Total
Suckling piglets (0-3 weeks)	5/ 26 (19.2%)	1/7 (14.2%)	6/33 (18.18%)
Weaning piglets (3-9 weeks)	15/ 57 (26.3%)	7/30 (23.3%)	22/87 (25.28%)
Fattening pigs (9-24 weeks)	1/28 (3.4%)	0/9 (0%)	1/37 (2.7%)
Adult pigs (>24 weeks)	0/16 (0%)	0/3 (0%)	0/19 (0%)
Total (%)	21/127 (16.53%)	8/49 (16.32%)	29/176 (16.47%)

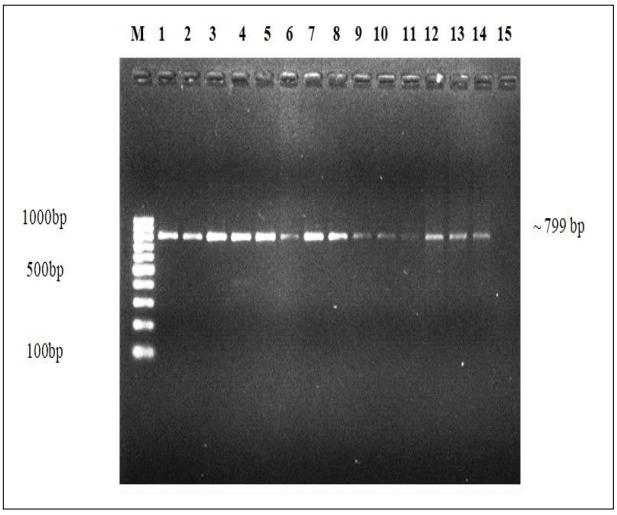
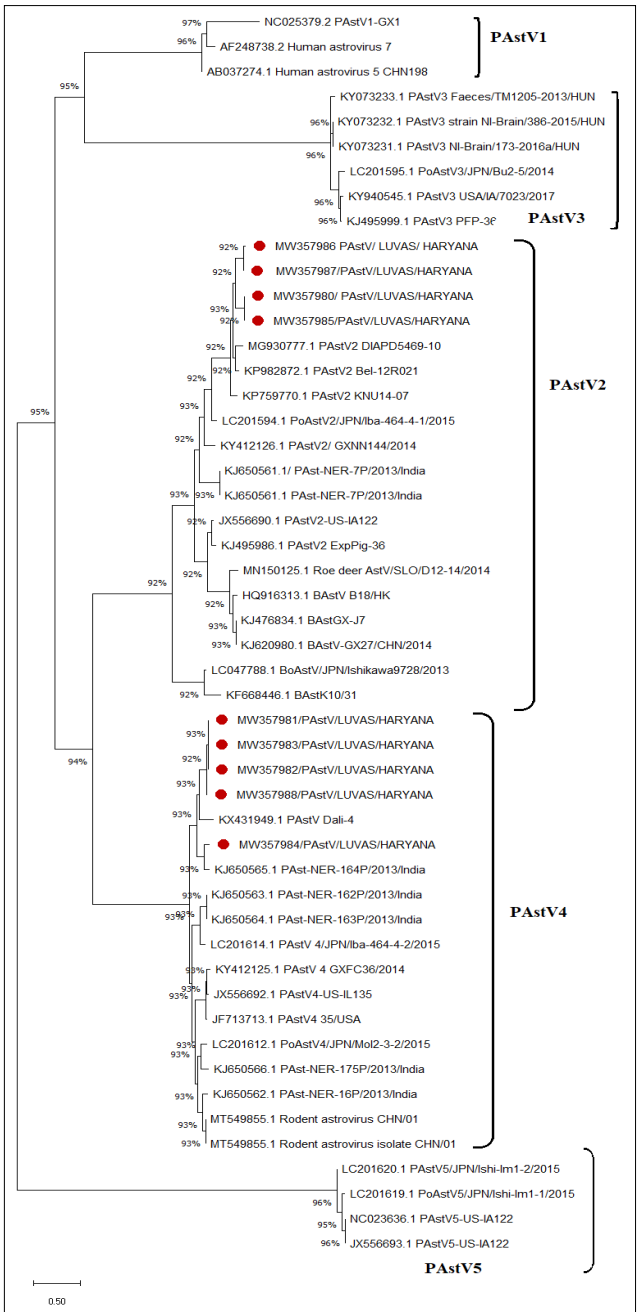


Fig 2: Agarose gel electrophoresis of PCR products of partial partial ORF1b/ORF2 region of PAstV.
Lane M: 100 bp ladder; Lane 1: Positive control; Lane 2-14: Samples; Lane 15: Negative control.

(Kattoor *et al*., 2019), 8.8% (25/129) in South Korea (Lee *et al*., 2013) and 16.4% (89/543) in China (Su *et al*., 2020). Other studies have found PAstV to be 31.8% in Haryana, India (Kaur *et al*., 2021), 34.2% (67/196) in the Czech Republic (Dufkova *et al*., 2013) and 62% (166/269) in the United States (Mor *et al*., 2012). The data in the present study revealed that there is notably no difference in the secretion of PAstV between diarrheic (16.53%) and non-diarrheic (16.32%) pigs (Table 1). PAstV was not found to be associated with diarrhea in some earlier reports (Lee *et al*., 2013; Cai *et al*., 2016; Kumthip *et al*., 2018; Salamunova *et al*., 2018). Further, the per cent positivity was higher in weaning piglets than in suckling piglets (Table 1). The high susceptibility of weaning piglets to viral diarrhea may be due to a declining maternal antibody titer and inefficient immune response (Amimo *et al*., 2014; Wang *et al*., 2006).

Out of the 29 PAstV positive samples, six from diarrheic and three from non-diarrheic pigs were commercially sequenced by the Sanger sequencing method. The obtained sequences were submitted to NCBI GenBank (MW357980 to MW357988). The phylogenetic analysis of the partial ORF1b/ORF2 nucleotide region revealed two lineages, *viz*., PAstV4 (5/9) and PAstV2 (4/9), circulating in Haryana (Fig 3). Similar findings were reported earlier from India and Thailand (Kumthip *et al*., 2018; Kattoor *et al*., 2019). Five nucleotide sequences from the present study (MW357981, MW357982, MW357983, MW357984 and MW357988) clustered within the PAstV4 group and were closely related to PAstV strains; KX431949 from China and LC201614 from Japan shared 77.5-81% and 71.9-75.5% nucleotide identities, respectively. Of the nucleotide sequences of PAstV4 group; MW357984 clustered along with the Indian strain, KJ650565 sharing 87.5% nucleotide identity. Per cent identity within PAstV4 nucleotide sequences from present study and previously reported Indian strains ranged from 70.3-78.8%. The remaining four PAstV sequences (MW357980, MW357985, MW357987 and MW357986) clustered with the PAstV2 lineage and were related to other PAstV strains; KP982872 from Belgium, MG930777 from Italy, KP759770 from South



GenBank accession numbers are shown on the tree and the sequences from Haryana, India are indicated by red dots.

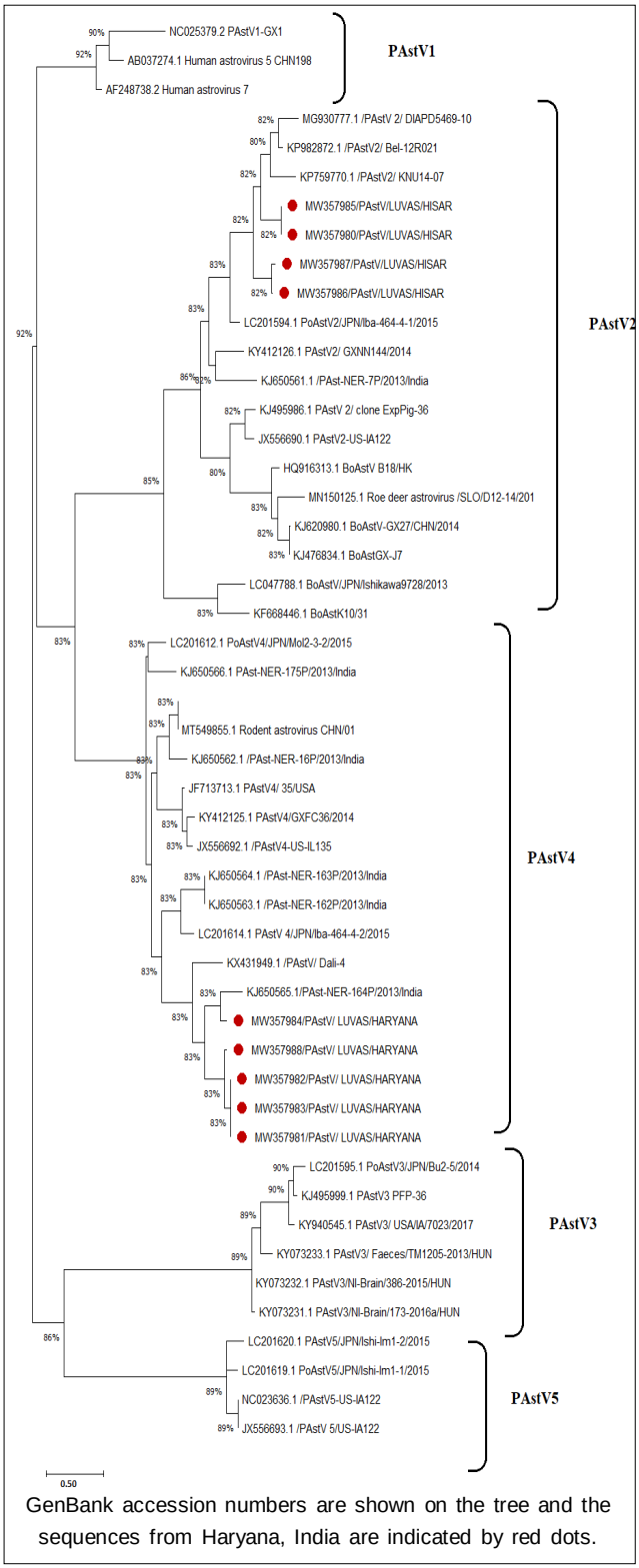
Fig 3: Phylogenetic analysis on the basis of partial ORF1b/ ORF2 nucleotide sequences using Maximum likelihood method and Tamura-Nei model with gamma distribution keeping the bootstrap value to 1000 replicates.

Korea and KJ650651 from India, which shared 83.6–85.3%, 79.3-84.8%, 80.5-83.5% and 69.4-71.0% nucleotide identities, respectively. Overall percent identity at nucleotide level among sequences from this study and other parts of India ranged from 69.4-78.8% which revealed the circulation of highly variable PAstV strains in India.

Phylogenetic analysis based on deduced amino acid sequences of the partial ORF2 gene segment also revealed the presence of PAstV4 and PAstV2 lineages in Haryana (Fig 4). In the PAstV4 clade, same five sequences (MW357981, MW357982, MW357983, MW357984 and MW357988) were clustered, sharing amino acid identities of 50.3-81.8% and 43.0-68.5% with PAstV strains; KX431949 from China and LC201614 from Japan, respectively. Furthermore, the amino acid identities with previously reported Indian PAstV4 strains ranged from 41.3 to 74.1%. The remaining four sequences in the PAstV2 clade (MW357980, MW357985, MW357987 and MW357986) shared amino acid identities of 82.6-91.7% with KP982872 from Belgium, 79.7-83.3% with MG930777 from Italy, 79- 83.9% with KP759770 from South Korea and 46.55-50%with KJ650561 from India. These findings suggested that the ORF2 region, which codes for capsid protein is more divergent as compared to the ORF1b gene coding for viral polymerase (Arias and DuBois, 2017). Furthermore, clustering of amino acid sequences of rodent AstV with the PAstV4 lineage, bovine and deer AstV with the PAstV2 lineage and human AstV with the PAstV1 lineage was observed, implying multiple recombination events or interspecies transmission in the past (Lukashov *et al*., 2002; Xiao *et al*., 2013; Ito *et al*., 2017).

Briefly, in the present study, ORF1b/ORF2 region sequences of nine PAstV shared 22.7-100% nucleotide identity and 79.9-100% amino acid identity based on ORF2 region. Similarly earlier studies from India have revealed 16.9-99.2% (Kattoor *et al*., 2019) and 31.5- 100% nucleotide identities (Kaur *et al*., 2021). In the present study, the percent nucleotide identities within the PAstV4 and PAstV2 lineages were found to range between 60.1-100% and 79.9-100%, respectively whereas the amino acid identities within PAstV4 and PAstV2 sequences ranged between79.1-100% and 81.1-100%, respectively. Hence, from the present analysis, it can be concluded that a wider range of genetic variation is present in PAstVs, especially in ORF2 region which is a common feature of the family *Astroviridae* (Strain *et al*., 2008; Lv *et al*., 2019; Wohlgemuth *et al*., 2019). The ORF2 encodes for a capsid protein containing various immunogenic epitopes that are under positive selection pressure. As a result, there are opportunities for evolution, such as interspecies transmission and recombination (Wohlgemuth *et al*., 2019).

Additionally, nucleotide sequences in this study have a common conserved region: “UUUGGAGGGG (A/C) GGACCAAAN (11) AUG GC” (where N stands for any of 4 nucleotides) at the overlapping region of ORF1b/ORF2 gene just before the start codon of ORF2 gene (Fig 5). These



GenBank accession numbers are shown on the tree and the sequences from Haryana, India are indicated by red dots.

Fig 4: Phylogenetic analysis using/based onpartial ORF2 amino acid sequences using maximum likelihood method and Jones-Taylor-Thornton (JTT) method with Gamma distribution and keeping the bootstrap value to 1000 replicates.

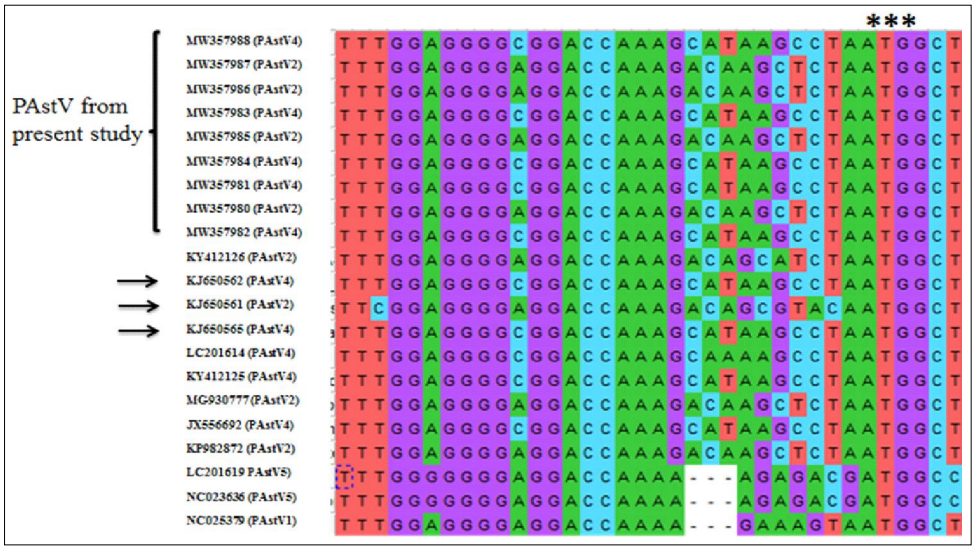


Fig 5: Conserved nucleotide sequence at the ORF1b/ORF2 junction upstream the start codon (ATG) of ORF2 represented by asterisks. The black arrows indicate the PAstV strains of Indian origin.

findings are consistent with earlier reports revealing the presence of similar conserved region with N (11) (Xiao *et al*., 2017; Qin *et al*., 2019) compared to N (4-8) in other studies (Lan *et al*., 2011; Luo *et al*., 2011). This conserved region is hypothesized to act as a regulatory element, serving as a promoter for sub-genomic RNA transcription (Qin *et al*., 2019). The significance of the change in length of regulatory element in the viral genome needs further study.

CONCLUSION

In the present study, frequency of detection was found to be 16.47% in both healthy and diarrheic pigs, suggesting no association between the diarrheic status of pigs and detection of this virus by RT-PCR. The results signify that the distribution of PAstV varies widely among different geographical regions. The weaning piglets were the most affected age group with PAstV infection followed by suckling piglets and fattening pigs. The phylogenetic analysis revealed the circulation of PAstV2 and PAstV4 lineages in Haryana, India. Further studies are required to be conducted with more samples collected from a larger geographical area to determine the role of the PAstV in causing diarrhea among pigs and its molecular characterization. The whole-genome sequences from the area under study would be helpful to study the genetic diversity between the PAstVs and possible recombination events for understanding viral evolution.

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Author's contribution

Rajpreet Kaur, Parveen Kumar, Naresh Jindal, Sanjeevna K. Minhas designed the work; Rajpreet Kaur and Parveen Kumar collected the samples; Rajpreet Kaur, Parveen Kumar, Sanjeevna K. Minhas, Rajpreet Kaur and Deepika Sheoran performed the research work; Rajpreet Kaur, Parveen Kumar, Sanjeevna K. Minhas, Anand Prakash and Vinay Joshi analyzed the results; Rajpreet Kaur, Parveen Kumar, Naresh Jindal, Sanjeevna K. Minhas, Naresh Kumar, prepared the manuscript.

Code or data availability

The dataset generated during the current study has been deposited in GenBank with the accession numbers MW357980 to MW357988.

Consent for publication

All authors gave their consent for publication.

Conflict of interest

The authors declare no conflict of interest.

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