Molecular Detection and Characterization of Porcine Astrovirus on the basis of Partial ORF1b/ORF2 Region

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Abstract

The porcine astrovirus (PAstV) is distributed globally and exists as five distinct lineages (PAstV1-PAstV5). PAstV is considered one of the important pathogen associated with diarrhea among pigs. In the present study, the PAstV was detected in 13.4% (19/141) of fecal samples including 14.4% (16/111) diarrheic and 10% (3/30) non-diarrheic samples by RT-PCR based on partial ORF1b/ORF2 gene from Haryana, India. The results indicated that the weaning piglets were more susceptible to PAstV infection followed by suckling piglets. The phylogenetic analysis of the viral strains revealed the circulation of PAstV4 (55.5%) and PAstV2 (44.4%) lineages with PAstV4 being the predominant lineage. To conclude, RT-PCR screening followed by sequencing of PAstV revealed high genetic diversity among the PAstV strains suggesting the wide range of heterogeneity and possible recombination events of viral strains in the state.

Introduction

Porcine astroviruses (PAstVs) are widely distributed and highly prevalent viruses in pigs. PAstV belongs to the family *Astroviridae* which comprises of two genera, *Mamastrovirus* and *Avastrovirus* isolated from mammals and birds respectively. The PAstVs have been further classified into five distinct lineages (PAstV1-PAstV5) based on the phylogenetic analysis of the ORF1b and ORF2 sequences (Lee et al., 2013; Qin et al., 2019). PAstV is a spherical, non-enveloped virus with a distinctive star-like morphology and about 28-30 nm size. The virus contains a single-stranded positive-sense RNA with genomic length of about 6.4–7.3 kb (Fang et al., 2019). The genome is divided into three open reading frames (ORFs) termed as ORF1a, ORF1b and ORF2 flanked by untranslated regions (UTRs) at both 5' and 3' end of the genome. The ORF1a and ORF1b encode for non-structural proteins including serine protease, viral genome linked protein (VPg), three to five transmembrane helices and RNA dependent RNA polymerase (RdRp) respectively which are required for viral replication and transcription (Zhao et al., 2019). The ORF2 encodes for structural capsid proteins, which are involved in viral assembly and form the neutralizing epitopes and spike proteins that are helpful in adhesion of the virus to host cell membrane (Ito et al., 2017).

PAstVs are primarily enteropathogens and are associated with diarrhea in pigs of all age groups. The pigs infected with PAstV show anorexia, diarrhea and failure to thrive (Moser and Schultz-Cherry 2005). However, PAstV has also been detected in pigs showing no signs of diarrhea (Kumthip et al., 2018; Qin *et al.*, 2019). In addition, PAstV has also been associated with respiratory infections, encephalitis and disseminated infections signifying greater clinical importance of the virus (Xiao et al., 2013; Padmanabhan and Hause 2016; Boros et al., 2017)

PAstVs are distributed worldwide and are endemic in several countries of Europe and North America and less common in Asian countries (Kumthip et al., 2018). In a study conducted in India, fecal samples collected from ten different states revealed the presence of PAstV in 17.6% (133/757) of samples with the circulation of PAstV4 and PAstV2 lineage (Kattoor et al., 2019). In a preliminary study conducted in Haryana, PAstV was detected in 31.8% (35/110) diarrheic pigs using the partial ORF1b gene with the

circulation of multiple lineages (Kour et al., 2021). However, these studies have not covered the infection status in healthy pigs in the region. Therefore, to understand the circulation of PAsTV in diarrheic and healthy pigs the present survey was conducted. The survey was conducted in both healthy and diarrheic pig samples to detect PAsTV infection in fecal samples collected from different parts of Haryana, a northern state of India, by RT-PCR followed by genetic typing and molecular characterization of the virus based on partial ORF1b/ORF2 region with other known astrovirus.

Sample Collection And Detection Of PAsTV

From September 2019 to February 2020, a total of 141 rectal swabs were collected from 11 piggery units located in different parts of Haryana, a northern state of India. The samples were collected from both diarrheic (n=111) and clinically healthy pigs (n= 30) of different age groups. A total of 26 samples were collected from suckling pigs (0-3 weeks), 61 samples from weaning pigs (3-9 weeks), 35 samples from fattening pigs (9-24 weeks) and 19 samples from adult pigs (> 24 weeks).

The sampling procedure, viral RNA purification and cDNA synthesis were as described previously (Kour et al., 2021). Due to the non-availability of positive control, a PAsTV positive PCR product was cloned, sequenced, confirmed by bioinformatics tools, and then used as a positive control for detecting PAsTV in rectal swabs using RT-PCR. After screening six samples, PAsTV/LUVAS/Haryana/04 tested positive by PCR of ORF1b/ORF2 gene specific primers which was purified, cloned and transformed into TOP10F' *E.coli* competent cell as described previously (Kour et al., 2021). The recombinant plasmids were isolated using GeneJET Plasmid Miniprep Kit (Thermo Scientific, U.S.A) and confirmed by PCR, RE digestion and sequencing. The RE digestion of the recombinant plasmid containing partial ORF1a/ORF2 gene insert (pJPAsTV799) was set up using 10X CutSmart buffer (NEB, UK) in 30 µl reaction volume, using *Xba*I and *Not*I restriction enzymes (NEB, UK). The reaction mixture was incubated at 37°C for 2h and confirmed using 1.0% agarose gel. The RE digested product showed two bands of the vector backbone (~2.97kb) and targeted gene size of ~ 799 bp. The recombinant plasmid was further confirmed by Sanger sequencing and used as positive control.

All the samples were then screened by RT-PCR using partial ORF1b/ORF2 region primers and the recombinant plasmid, pJPAsTV799 was used as positive control. The PCR was performed using primer pair consisting of forward primer PAsTV1F (5'-TGACATTTTGTGGATTACAGTT-3') and reverse primer PAsTV1R (5'-CACCCAGGGCTGACCA-3') yielding an expected size of approximately 799 bp PCR product (Kumthip et al., 2018). The PCR was carried out using 10 µl of 2X Dream Taq green PCR mastermix (Thermo Scientific, U.S.A), 0.6 µl of each 10 µM forward and reverse primer (PAsTV1F and PAsTV1R), 2 µl cDNA and nuclease-free water to make up 20 µl of the total reaction volume. Amplification conditions were optimized at initial denaturation of 95°C for 4 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min and final extension at 72°C for 7 min. The PCR products were confirmed by electrophoresis in 1.5% agarose gel and visualized by Gel Documentation system (Azure Biosystem, U.S.A).

Nucleotide Sequencing And Phylogenetic Analysis

The virus positive PCR products were gel purified using a QIAquick gel extraction kit (Qiagen, Germany). The purified products were sequenced commercially in both directions using Sanger's sequencing method. The chromatogram data received was initially checked using FinchTV software and the final sequences were generated with the help of MEGA X software. These nucleotide sequences were phylogenetically compared with different nucleotide sequences of human AstV, rodent AstV, bovine AstV, deer AstV and other PAstV retrieved from the NCBI database. The sequences were aligned using the Clustal W program in MEGA X software and a phylogenetic tree was constructed using maximum likelihood method and Tamura-Nei model with gamma distribution keeping bootstrap value to 1000 replicates.

Further, the nucleotide sequences of the partial ORF2 region were translated and the aligned amino acid sequences were used for the construction of the phylogenetic tree using the best fit model selected by MEGA X software. The phylogenetic tree was constructed using 150 amino acids sequence by maximum likelihood method and Jones-Taylor-Thornton (JTT) method with Gamma distribution and keeping the bootstrap value to 1000 replicates.

Results And Discussion

In the present study, 19 (13.4%) of the 141 rectal swab samples tested positive for PAstV by RT-PCR of partial ORF1b/ORF2 region. Of the positive samples, 16/111 (14.4%) were from diarrheic pigs and 3/30 (10%) were from non-diarrheic pigs. Since PAstV was detected in both healthy and diarrheic pigs, no clear association of PAstV infection with diarrhea could be ruled out. Our results concur with earlier reports showing 17.6% occurrence of PAstV from ten different states of India (Kattoor et al., 2019). Whereas in an earlier study from Haryana using partial ORF1b gene primers, 31.8% of samples tested positive for PAstV. The difference in the results could be due to the difference in the efficiency of primers used and the high genetic variability in the ORF2 region (Kour et al., 2021).

Among the positive samples, the percent positivity was more in weaning piglets followed by suckling piglets (Table 1). The weaning piglets are more susceptible to viral diarrhea possibly due to a decline in maternal antibody titer and inefficient immune response making them more prone to infections (Wang et al., 2006; Amimo et al., 2014).

Table 1
Age wise distribution of PAsV in the samples collected from both diarrheic and non-diarrheic pigs of Haryana, India

Age categories	Diarrheic pigs	Non-diarrheic pigs	Total
Suckling piglets (0-3 weeks)	4/ 22 (18.1%)	0/4 (0%)	4/26 (15.3%)
Weaning piglets (3-9 weeks)	11/ 45 (24.4%)	3/16 (18.7%)	14/61 (22.9%)
Fattening pigs (9-24 weeks)	1/28 (3.5%)	0/7 (0%)	1/35 (2.8%)
Adult pigs (>24 weeks)	0/16 (0%)	0/3 (0%)	0/19 (0%)
Total	16/111 (14.4%)	3/30 (10%)	19/141 (13.4%)

Out of the 19 positive samples, 9 were commercially sequenced by the Sanger sequencing method. The obtained sequences were submitted to NCBI GenBank and accession numbers MW357980 to MW357988 were assigned.

On phylogenetic analysis of the partial ORF1b/ORF2 nucleotide region, two lineages *viz*, PAsV4 (5/9) and PAsV2 (4/9) were found circulating in Haryana with PAsV4 (55.5%) being predominant (Fig. 1). These findings concur with the earlier study from India, where PAsV4 (21/22) and PAsV2 (1/22) were reported in India with PAsV4 being predominant (Kattoor et al., 2019). Similarly, the lineage PAsV4 and PAsV2 were also detected in Thailand with PAsV4 being predominant (Kumthip et al., 2018) using the same primer set.

Five nucleotide sequences from the present study (MW357981, MW357982, MW357983, MW357984 and MW357988) clustered within PAsV4 group and were closely related to PAsV strain; KX431949 from China and LC201614 from Japan sharing 77.5–81% and 71.9–75.5% nucleotide identities, respectively. In addition to these one of the nucleotide sequences; MW357984 clustered more closely with the Indian strain, KJ650565 with 87.5% nucleotide identity. However, the nucleotide identities of all the PAsV4 sequences from the present study with previously reported PAsV4 Indian strains ranged from 70.3–78.8%. The remaining four PAsV strains (MW357980, MW357985, MW357987 and MW357986) from this study clustered with PAsV2 group and were related to PAsV strains; KP982872 from Belgium sharing 83.6–85.3% nucleotide identity, MG930777 from Italy sharing 79.3–84.8% nucleotide identity and KP759770 from South Korea sharing 80.5–83.5% nucleotide identity. Moreover, the nucleotide identities with previously reported PAsV2 strain, KJ650651 from India ranged from 69.4–71.0%.

Phylogenetic analysis based on the amino acid sequence of the partial ORF2 gene segment also revealed the presence of two lineages; PAsV4 and PAsV2 circulating in Haryana (Fig. 2). In the PAsV4 clade, same five sequences were clustered sharing amino acid identities of 50.3–81.8% and 43.0–68.5% with PAsV strain; KX431949 from China and LC201614 from Japan, respectively. Furthermore, the amino acid identities with previously reported PAsV4 Indian strains ranged from 41.3–74.1%. The remaining four sequences in the PAsV2 clade shared amino acid identities of 82.6–91.7% with KP982872 from Belgium, 79.7–83.3% with MG930777 from Italy and 79–83.9% with KP759770 from South Korea. The amino acid identities with the India isolate, KJ650561 ranged from 46.55 to 50%.

The sequences of rodent AsV clustered along with PAsV4 group whereas the sequences of bovine AsV and the deer AsV clustered along with PAsV2 group. The sequences of the human AsV clustered within PAsV1 group. This could possibly be due to the multiple recombination events or interspecies transmissions in the past (Lukashov and Goudsmit 2002; Xiao et al., 2013)

The nucleotide sequences of the partial ORF1b/ORF2 region of samples processed in the present study have sequence identities ranging from 22.7–100% at the nucleotide level and 79.9–100% at the amino acid level, respectively. Similar results have been reported from India earlier showing nucleotide identities ranging between 16.9%-99.2% (Kattoor et al., 2019) and 31.5% - 100% (Kour *et al.*, 2020) The percent identity within PAsV4 and PAsV2 lineages was found to range between 79.1%-100% and 81.1%-100% respectively based on nucleotide sequences. The amino acid identity within PAsV4 and PAsV2 sequences ranged between 60.1–100% and 79.9–100% respectively. Hence from the present analysis, it can be concluded that a wider range of genetic variation is present in PAsVs which is a common feature of the family *Astroviridae* (Wohlgemuth et al., 2019). However, it was observed that the range of divergence is more when comparing the sequences based on partial ORF2 amino acid region, and this might be due to high variability in the ORF2 region of the PAsV genome (Strain et al., 2008).

Besides this the sequence analysis by MEGA X software revealed the presence of this conserved nucleotide sequence; “UUUGGAGGGG (A/C) GGACCAAA N(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. 3) in all the 9 sequences. This conserved region is hypothesized to act as a regulatory element, serving as a promoter for sub-genomic RNA transcription (Lv *et al.*, 2019; Qin et al., 2019). The sequence comparison indicated that there is a presence of N (11) in the samples sequenced in the present study instead of N(4-8), as reported earlier (Xiao et al., 2013).

Conclusion

This study reports the presence of PAsV in 13.4% of the pigs including both healthy and diarrheic pigs with more percent positivity in diarrheic pigs than non-diarrheic pigs. The results signify that the distribution of PAsV varies widely among different geographical regions. The weaning piglets are the most affected age group with PAsV infection. The phylogenetic analysis revealed the circulation of PAsV2 and PAsV4 in Haryana with PAsV4 being more predominant. High genetic variability was

observed among the circulating viral strains showing the heterogeneity of PAsV present in a limited area underlining the need of molecular characterization of PAsV. Further studies are required to determine the role of the PAsV in causing diarrhea among pigs. A large sample size and whole-genome sequences will be required to study the role of PAsV in causing diarrhea and to study the genetic diversity among the PAsV so that appropriate control strategies can be implemented.

Declarations

Authors' Contribution:

RK, PK and NJ designed the research work. RK, PK and SKM performed the research work. RK, PK, NJ, SKM and VGJ analyzed the result. RK, PK, NJ, SKM, AP, SD, NK and VGJ prepared and edited manuscript.

Ethical approval:

Not required.

Code or data availability:

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

Consent for participation:

Not applicable.

Consent for publication:

All authors consent for publication.

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Figures

Figure 1

Phylogenetic analysis on the basis of partial ORF1a/ORF2 nucleotide sequences using Maximum likelihood method and Tamura-Nei model with gamma distribution keeping the bootstrap value to 1000 replicates. GenBank accession numbers are shown on the tree and the sequences from Haryana, India are indicated by red dots. Blue squares represent sequences reported earlier from India

Figure 2

Phylogenetic analysis using/based on partial 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. GenBank accession numbers are shown on the tree and the sequences from Haryana, India are indicated by red dots. Blue squares represent sequences reported earlier from India

Figure 3

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