RESEARCH ARTICLE

Occurrence of G10P[11] Genotype of Group A Bovine Rotavirus in Diarrheic Calves from in and around Navsari, Gujarat

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Abstract

A study was undertaken to ascertain genotypic characterization of bovine group A rotavirus circulating in the Navsari region of Gujarat state. Total 157 diarrheic fecal samples were collected from bovine calves of organized farms and locally at village level from Navsari region. All the samples were initially screened by RNA-PAGE and RT-PCR followed by characterization by sequencing and G-P typing. The study revealed 10 (6.36%) samples positive by RNA-PAGE, while 16 (10.19%) samples were positive by VP4, VP6 and VP7 gene-based RT-PCR. From these positive samples, representative samples were sequenced for complete coverage of VP4, VP6, and VP7 genes followed by P and G typing on the basis of VP4 and VP7 sequence results, respectively. All the sequenced samples revealed G10 and P[11] types, showing a G10P[11] combination significantly prevailing in the bovine calves. Phylogenetic analysis of VP4, VP6, and VP7 genes was performed to determine the cross-species transmission dynamics and regional diversity, which showed similarity with other isolates circulating in India. Our findings suggested that a zoonotic potential genotype G10P[11] is the most circulating genotypes of Rotavirus A in bovine calves from Navsari region of Gujarat, India.

Key words: Bovine calves, Genotyping, G-P typing, Rotavirus A, RNA-PAGE, RT-PCR.

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Introduction

Livestock farming is one of the significant sectors in India and it plays an important role in economy of the country. Several factors cause economic losses to the calves farming; among them, neonatal calf diseases like diarrhea (scours) and respiratory diseases are the leading causes (George *et al*., 2010). Rotaviruses have triple-layered capsid and segmented dsRNA genome (Deshmukh *et al*., 2016). Rotaviruses (RVs) are classified into seven groups (A, B, C, D, E, F, and G) as per the antigenic variability of the inner capsid protein VP6. Based on outer capsid proteins VP4 and VP7, group A rotaviruses is further classified into P (VP4) and G (VP7) types, respectively. Among different groups of rotaviruses, Group A rotaviruses have been found to be predominantly present in cases of diarrheic domesticated animals and avian species (Niture *et al*., 2011). In different studies, BRV infection rates of 20–60% in samples of diarrhea have been reported. Prevalence of rotavirus ranges from 11.8% to 26.8% in India among diarrheic calves (Geletu *et al*., 2021). To prevent the spread of rotavirus and accurate diagnosis nucleic acid based techniques like PCR and RNA – PAGE are promising tests as it confirms the presence of virus and these tests are less laborious and more convenient compared to cell culture techniques.

Due to the re-assortment of genomic segments (genetic shift), rotaviruses exhibit high genetic diversity (Matthijnssens *et al*., 2012) and reassortment in the case of mixed infection in natural conditions leads to the emergence of new serotypes of the virus (Niture *et al*., 2011). In bovines, the most frequently observed types are G6, G8, and G10 and P[1], P[5], and P[11] (Fukai *et al*., 2002). From an epidemiological perspective, genotyping of bovine rotaviruses is the most essential finding to regulate vaccination programs (Sravani

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et al., 2014). G and P typing has been extensively used for many years for assessment of genomic diversity and interspecies transmission of rotaviruses. Regular screening of fecal samples for detection of rotavirus A in a particular geographical area is vital to identify the circulating rotaviruses in susceptible populations. It is also important from the point of view of ecology and public health, because interspecies transmission from cattle to humans and from humans to cattle have been reported. Particularly rotavirus P[11], G10 strains, which are commonly found in cattle and reported from different geographical areas worldwide (Saravanan *et al*., 2007).- AS per VA1

Hence present study was carried out to check circularity of group A bovine rotavirus, their genotyping and molecular characterization in diarrhoeic calves from Navsari. This study reports the occurrence of G10P[11] genotype of group A bovine rotavirus in diarrheic calves from in and around Navsari, Gujarat.

Materials and Methods

Collection of Samples and Processing

Total of 157 diarrheic samples were collected from the rectum of bovine calves (up to 45 days of age) from different villages, organized farms and places in and around Navsari region, Gujarat (India). A 10% fecal suspension was prepared using phosphate buffer saline (PH 7.4), followed by centrifugation at 10,000 g for 20 min at 4°C (Heraeus Biofuge stratos centrifuge, Thermo Scientific) to remove coarse particles. The clarified supernatant was transferred to an RNAse free vial (5.0 mL, Eppendorf) and used for RNA extraction.

Detection of Rotavirus in Fecal Samples

All the fecal samples were screened by RNA-PAGE for the presence of rotavirus, followed by RT-PCR.

Ribonucleic Acid Polyacrylamide Gel Electrophoresis (RNA-PAGE): RNA-PAGE was used as pre-screening of the samples followed by RT-PCR. Viral RNA was extracted from the 10% fecal suspensions by the Trizol method. The segmented RNA genome of the rotavirus was analyzed by RNA-PAGE using the discontinuous buffer system without sodium dodecyl sulfate (SDS) as described by Laemmli (1970). Vertical gel electrophoresis was performed using an 8.0% separating gel and a 5.0% stacking gel. Samples were mixed with 6x loading dye and loaded into wells of the gel and subjected to electrophoresis. The gel was run at 100V till the dye ran out of the gel. The segmented genomic RNA was detected after staining of the gel by the silver-staining method (Herring *et al*., 1982).

Detection of Rotavirus by Reverse Transcriptase-PCR (RT-PCR): For detection of VP4, VP6, and VP7 genes, RNA was extracted from 10% fecal suspension by the QIAamp® Viral RNA Mini kit (Qiagen Sciences, MD, USA). Extracted genomic RNA was quantified by Nanodrop at the ratio of 260/280 and recorded. VP4, VP6, and VP7 genes of rotavirus were detected by reverse transcription PCR (RT-PCR) technique by employing one-step RT-PCR kit (SuperScript™ III one-step RT-PCR kit, Invitrogen). Briefly, 3 µL of genomic RNA was denatured at 95°C in a thermal cycler for 5 min and immediately chilled on ice. After this, the reaction mixture was prepared to a final volume of 25 µL as per the kit instructions. The VP4, VP6 and VP7 genes were amplified by previously published primers as per Table 1 and PCR cycling condition was set as per the kit instructions (Table 2). The amplified PCR products were analyzed by 1.5% agarose gel electrophoresis. The gel was visualized using the Gel documentation system (SynGene, UK).

Sequencing and Genotyping of Bovine Rotavirus A

Sequencing of VP4, VP6, and VP7 genes were done by sequencing primers according to previously reported study (Ramani *et al.,* 2009) for full coverage and the amplicons were purified using the PureLink™ Quick PCR purification kit (Invitrogen) and sent for Sanger sequencing (Eurofins Genomics India Pvt., Ltd., Bengaluru). Sequencing results were analyzed by the chromatogram analysis, BLAST on the NCBI database and alignment was done by CLUSTAL W software. Once validated, the sequences were formatted in

Table 1: List of primers used in RT-PCR for VP6, VP4 & VP7 genes

Gene	Primers sequence (5´–3´)	Amplicon size	Reference
VP6 (F)	GAC GGV GCR ACT ACA TGG T	379 bp	Falcone *et al*. (1999)
VP6 (R)	GTC CAA TTC ATN CCT GGT GG		
VP4 (Bov4Com5)	TTCATTATTGGGACGATTCACA	864 bp	Isegawa *et al.* (1993)
VP4 (Rev Bov4Com3)	CAACCGCAGCTGATATATCATC		
VP7-Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1062 bp	Gouvea *et al*. (1990)
VP7-End9	GGTCACATCATACAATTCTAATCTAAG		

Table 2: Thermal cycler condition for RT PCR

cDNA synthesis and pre-denaturation	Denaturation	Annealing temp according to primer		Extension	Final extension
1 cycle		35 Cycles			1 cycle
		VP4	48°C		
55° C	94°C	VP6	47°C	68°C	68°C
		VP7	43°C		
30 min	2 min	15 sec	30 sec	1 min	5 min

FASTA format and submitted to the NCBI database. Sequence results were submitted to the GenBank database with accession numbers OK318948, OK318949, and OK318950 for the VP7 gene; OK318951, OK318952, and OK318953 for the VP6 gene; and OK318954, OK318955, and OK318956 for the VP4 gene. The G and P genotypes were determined using the Rota C 2.0 genotyping tool.

Phylogenetic Analysis

The VP4, VP6, and VP7 sequence results of the present study along with other VP4, VP6, and VP7 sequences from India and other countries were retrieved from the NCBI database. A phylogeny was constructed by using the distance-matrix based Neighbor-Joining (NJ) method with a bootstrap value of 1000. The evolutionary distances were computed using the Kimura 2-parameter method and pair-wise deletion was used for the missing or gapped data by using MEGA X software.

Results and Discussion

Group A RVs are the leading cause of diarrhea in calves under one month of age throughout the world (Matthijnssens *et al*., 2012). As many infectious agents are responsible for neonatal calf diarrhea, confirmatory diagnostic tests are necessary for the detection of RV. The presence of an organism could only be recognized by a demonstrating organism by virus isolation or its nucleic acid (RNA) detection by RNA-PAGE or RT-PCR (Manuja *et al*., 2010). Both PAGE and RT-PCR are nucleic acid detection based reliable techniques.

Detection of Rotavirus A by RNA-PAGE

From 157 faecal samples of diarrheic calves, 10 samples (6.36 %) were found to be positive by RNA-PAGE analysis. All the positive samples showed a characteristic migration pattern of 4:2:3:2, which is a typical pattern of mammalian group A rotavirus. Electropherotyping of positive samples revealed two different migration patterns for group A RV (Fig. 1 & 2).

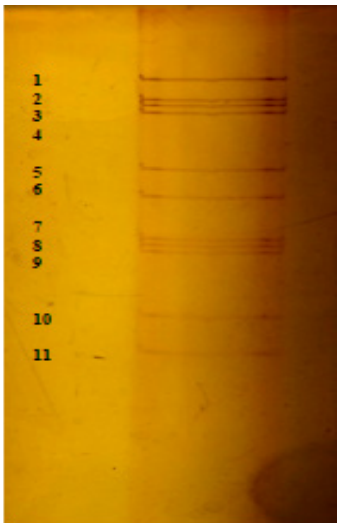

Fig. 1: Electropherogram pattern of positive sample Group A bovine rotavirus showing 4:2:3:2 migration pattern for 11 segments (Pattern I)

In the present study, all the isolates had long electropherotyping patterns. In a similar way, most researchers have found that long electrophoretic patterns are more common than short ones (Sravani *et al*., 2015; Kumari *et al*., 2019). The migration pattern of a particular RV is useful to analyze the genomic diversity and heterogeneity of the virus (Ambily *et al*., 2009). In accordance with present findings, others have also noted different migration patterns in their study (Basera *et al*., 2010; Kumari *et al*., 2019).

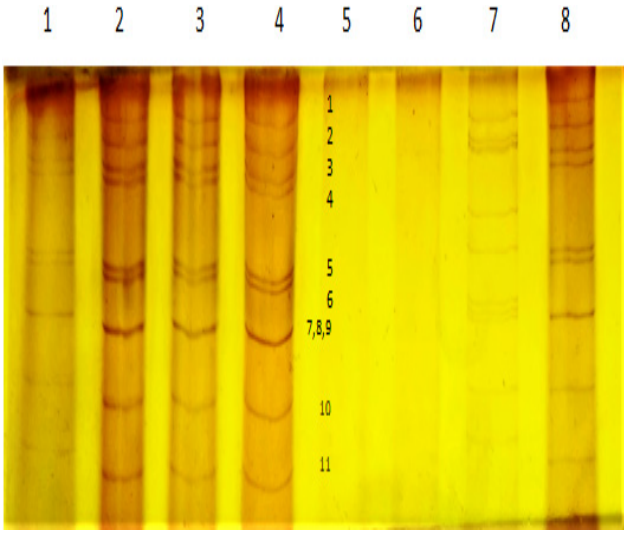

Fig. 2: Electropherogram migration pattern of group A bovine rotavirus samples of cow calves (Pattern II)

Detection of Rotavirus A by RT-PCR

Out of 157 samples, 16 were found positive for VP4, VP6 and VP7 genes that revealed 10.19% positivity for RVA. In RT-PCR, amplification of the VP4, VP6 and VP7 genes of RV could be achieved with a desired band size of 864 bp, 379 bp and 1062 bp, respectively (Fig. 3 & 4) in all the 16 samples. In this study, 16 samples were found positive by RT-PCR that is higher than RNA-PAGE by which 10 samples were confirmed for rotavirus. This result showed agreement with others (Ahmed *et al.,* 2017; Das *et al.,* 2018) who also detected higher numbers of positive samples by RT-PCR than RNA-PAGE. This is because of RT-PCR has the ability to detect 10^4 particles per gram of feces.

Sequencing, Genotyping, and Phylogenetic Tree of Bovine Rotavirus A

Out of 16 positive samples, three samples of cattle calves (B2, B4 and B5) were selected for VP4, VP6, and VP7 complete gene sequencing according to Ramani *et al.* (2009) (Fig. 5). Results retrieved after sequencing of VP7 and VP4 genes were used for G and P genotyping by the online RotaC 2.0 genotyping tool. This revealed G10 and P[11] in all three samples and showed circularity of G10P[11] in bovine calves of this region. Minakshi *et al.* (2005), Beg *et al.* (2010), Ahmed *et al.* (2017) also reported G10P[11] as the most predominant combination in the bovine rotaviruses. Contrary to this, Alfieri *et al.* (2004)

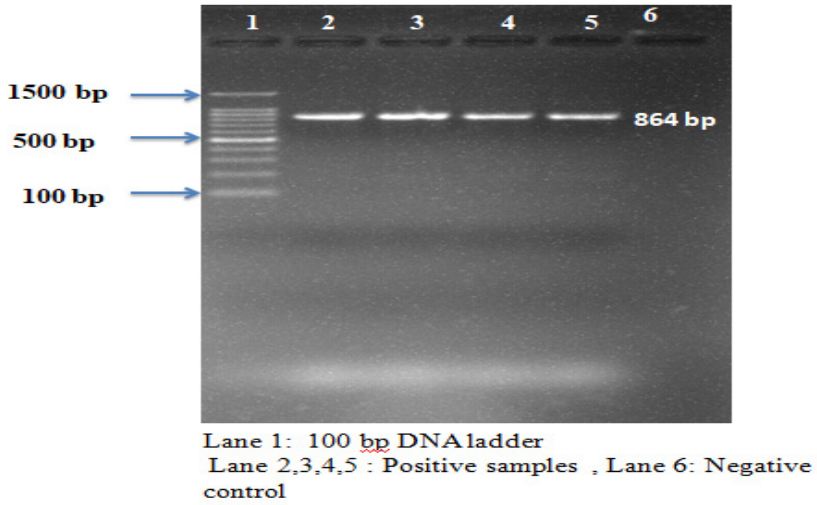

Fig. 3: Amplification of VP4 gene by RT PCR. Lane 1: 100 bp DNA ladder, Lane 2,3,4,5: Positive samples , Lane 6: Negative control

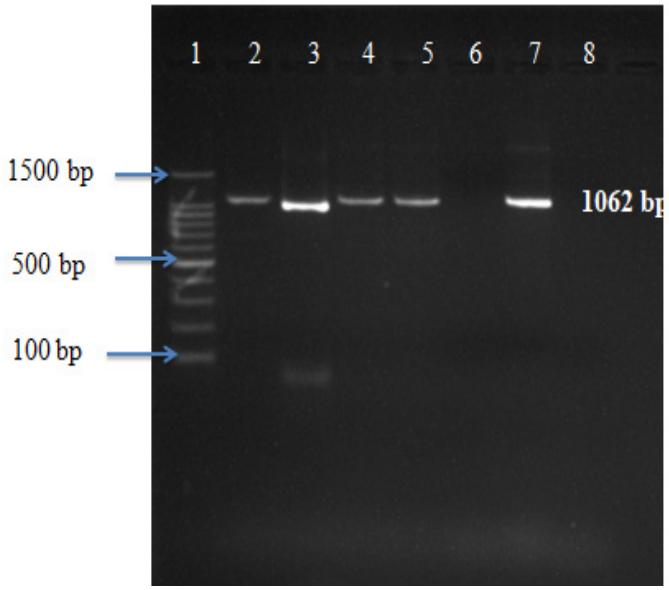

Fig. 4: Amplification of VP7 gene by RT PCR. Lane 1: 100 bp DNA ladder, Lane 2,3,4,5,7: Positive samples, Lane 8: Negative control, Lane 6: Negative sample

reported G6P [5] and Ghosh *et al.* (2007) reported G6P [11] combination in bovines.

The genotype G10P[11] is an important group A bovine rotavirus because of its zoonotic transmission from humans to cattle and also from cattle to humans (Iturriza-Gomara *et al.*, 2004). It has also been reported that G10P[11] strains are associated with symptomatic and asymptomatic infections in children in India (Hassine-Zaafrane *et al.*, 2014). Genome sequencing of Group A rotavirus strains has become the best way to figure out how strains are related genetically, and the function of each gene segment can be studied to figure out how rotaviruses have changed over time (Dhanze *et al.*, 2014).

Phylogenetic Tree of Genes VP4, VP6, and VP7

Sequence results of B2, B4 and B5 samples for VP4, VP6, and VP7 genes were subjected to NCBI BLAST analysis and confirmation for bovine RV. Phylogenetic analysis of VP4 genes of different isolates revealed separate clusters and similarities with different isolates. The VP4 gene of B2 showed similarities with JX442788.1 rotavirus isolated from the West Bengal, India whereas, VP4 gene of B4 showed similarities with EU311199.1 isolated from Punjab state of India. Surprisingly, VP4 gene of B5 grouped together with VP4 gene of human rotavirus isolated from Uttar Pradesh, India (Fig. 5). An analysis of the phylogeny of the VP6 gene showed that B2 and B4 shares similarities with KU292523.1, and VP6 gene of B5 also share similarities with MN066960.1, a rotavirus isolated from humans (Fig. 6). All three sequences have shown homology with rotavirus isolated from humans, though they were grouped separately but clustered together. Higher homology has been found

between the VP7 genes B2, B54 and B5. These sequences have shown similarity with the bovine rotavirus EF200547.1 and EF200543.1 which was found in Kolkata, West Bengal, India (Fig. 7).

Phylogenetic analysis of the present work indicated that the virus of this region showed similarity with rotavirus from other regions of India. In addition, it shows the possibility of virus circulation between different states of India as well as other countries. In addition, the samples showed homology with human and animal rotavirus sequences, which is indicative of interspecies transmission of rotavirus between humans and animals. This might be due to the poor hygienic condition of animal sheds and close contact between humans and livestock animals.

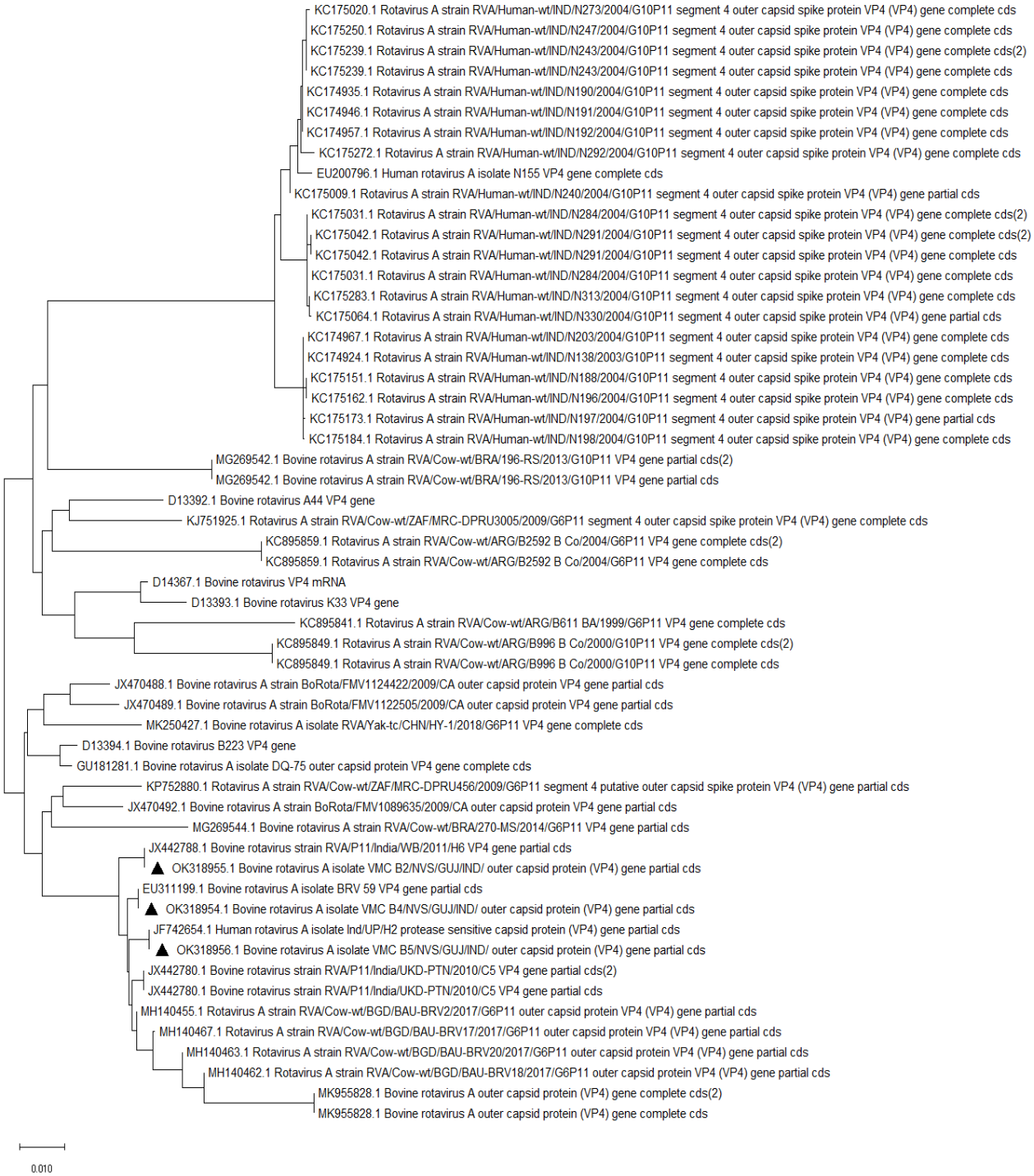

Fig. 5: VP4 gene nucleotide sequence based phylogenetic analysis of sequences of present study along with other isolates of group A rotavirus

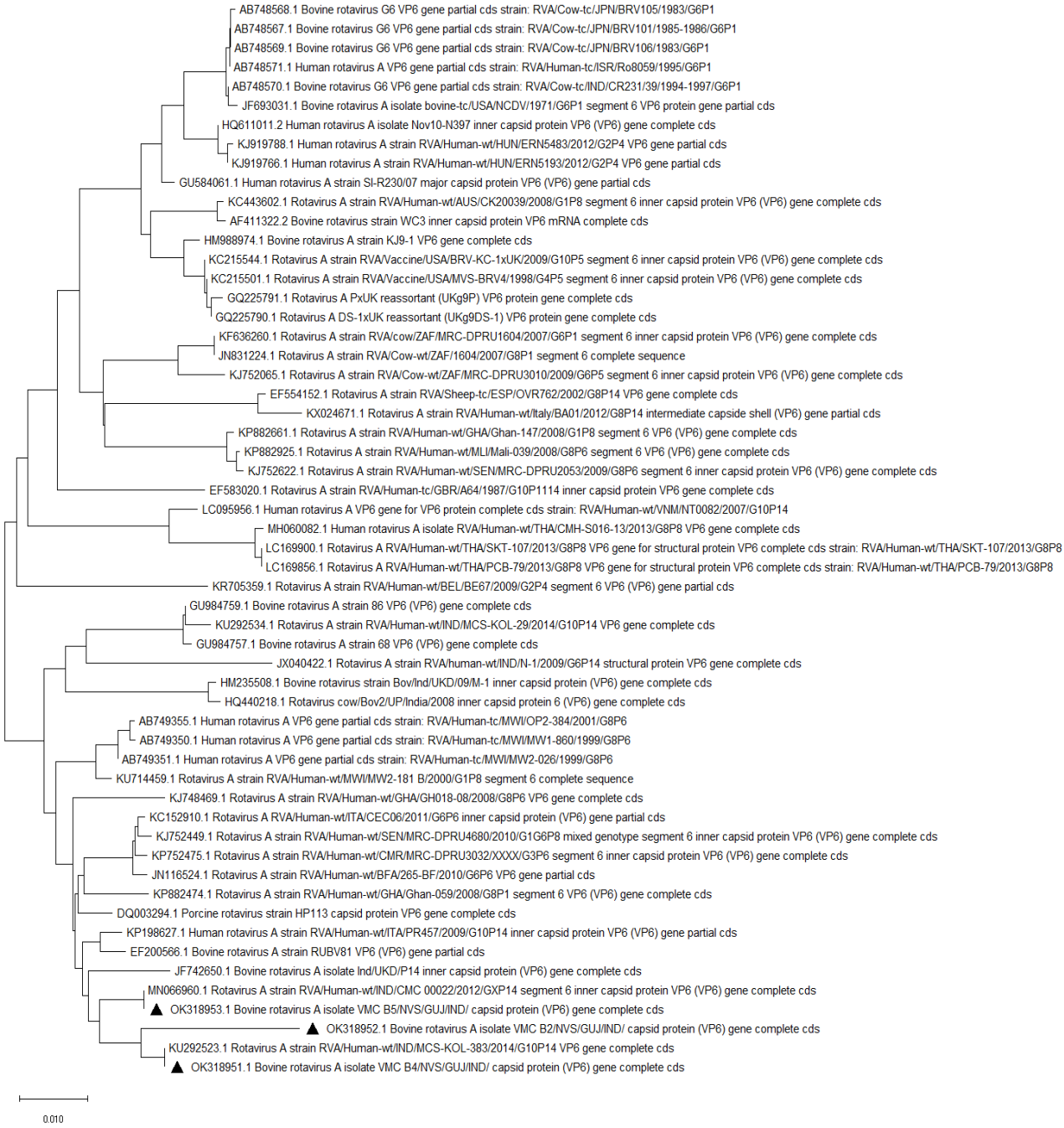

Fig. 6: VP6 gene nucleotide sequence based phylogenetic analysis of sequences of present study along with other global isolates of group A rotavirus

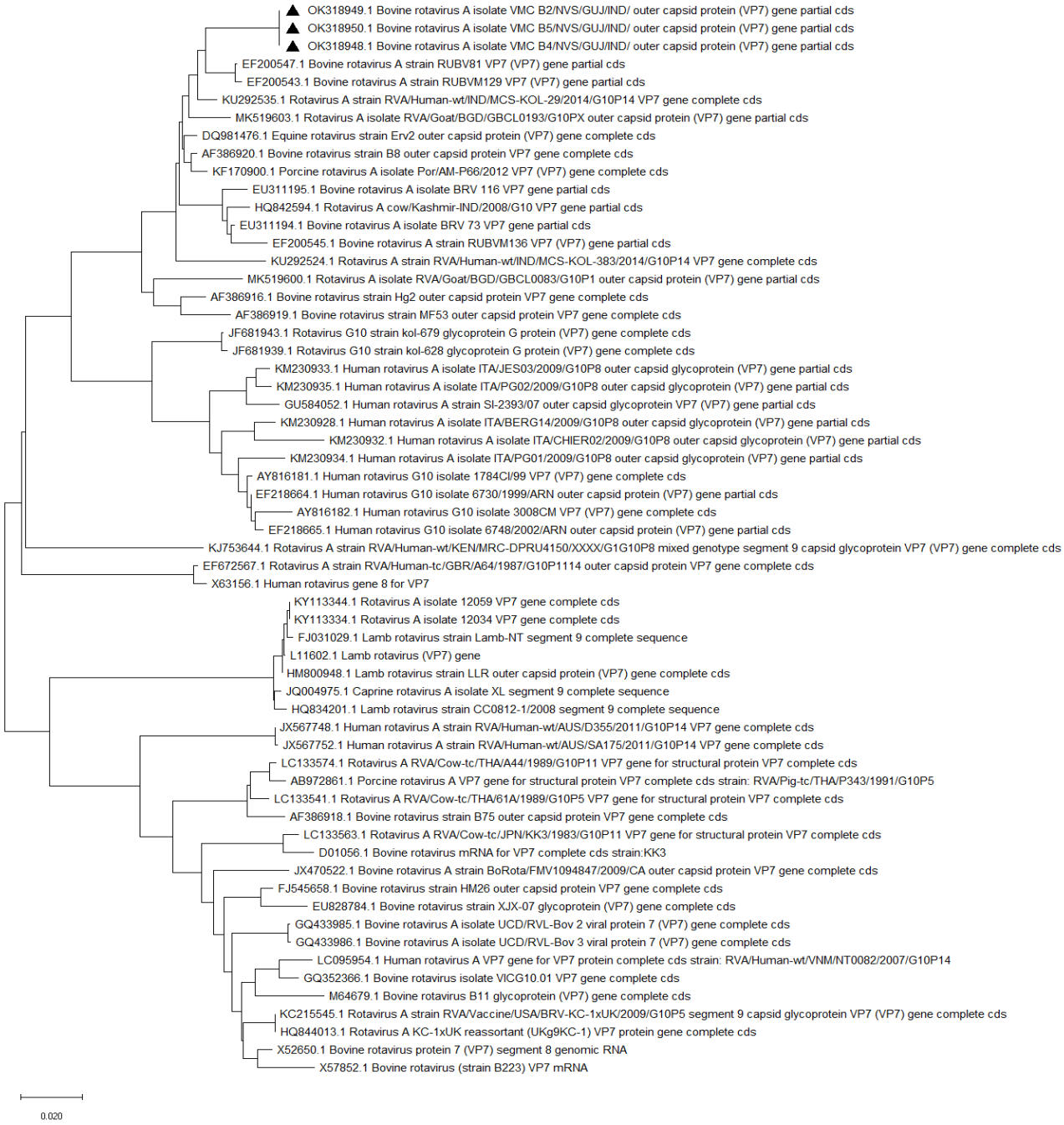

Fig. 7: VP7 gene nucleotide sequence based phylogenetic analysis of sequences of present study along with other global isolates of group A rotavirus

Conclusion

This study revealed circularity of G10P[11] genotype of rotavirus in bovine calves of Navsari region, Gujarat. RT- PCR was considered more sensitive technique as compared to RNA-PAGE in detection of rotavirus. G10P[11] is considered as zoonotic, so routine surveillance of rotaviruses in humans as well as animals is necessary.

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