Frequency distribution of porcine rotavirus-A and capsid protein gene based sequence and phylogenetic analysis indicating marked heterogeneity among prevailing strains, India

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Abstract Rotavirus (RV), is an etiological agent of acute infantile diarrhea in both humans and animals including poultry. Among the eight accepted species/types of RVs, RV-A is genetically and antigenically the most diverse. RV-A associated enteritis is a major problem in the weaning and post-weaning piglets. Due to high genetic variability in the antigenic regions, RV-A is thought to have high interspecies jumping probability. In this study, comparatively a large sized sample ($n = 757$) was screened, where the samples were collected from diarrheic porcine population of north (Uttar Pradesh), North eastern (Assam, Nagaland, Meghalaya, Tripura, Manipur,

Mizoram and Arunachal Pradesh) and Southern states of India (Kerala, Karnataka and Tamil Nadu). The VP6 gene based reverse-transcription (RT)-PCR based screening of the samples for RV-A identified 42.4% (321/757) positivity, where highest identification was from Uttar Pradesh 119 (37.07%), followed by 74 (23.05%), 34 (10.6%), 31 (9.65%), 21 (6.54%), 15 (4.67%), 11 (3.43%), 8 (2.49%), 3 (0.93%), 3 (0.93%) and 2 (0.62%) from Assam, Nagaland, Meghalaya, Tripura, Kerala, Manipur, Mizoram, Arunachal Pradesh, Karnataka and Tamil Nadu, respectively. Percentage identity calculation of the VP6 gene sequences from different porcine RV-A revealed 77.1–97.3% identity within the Indian porcine RV-A strains of the current study. Phylodendrogram and percent identity based analysis of the amplified and sequenced full length VP6 gene confirmed the presence of new VP6 genotypes (I1 and I5). Although, there are reports of detection of porcine RV-A based on VP6 gene from India, no lineage/genotype based characterization is available for the target gene. Till date, only a single VP6 type (I2) has been confirmed from pig population of India. Here, the findings confirm the circulation of diverse RV-A strains in porcine population in India.

Keywords Rotavirus · Porcine · Epidemiology · Sequencing · Phylogenetic analysis · VP6 gene · Genotyping · India

Introduction

Rotavirus (RV) is a typical member of the family *Reoviridae* and associated with colossal economic loss worldwide due to acute gastroenteritis. As on date, RVs are differentiated into eight accepted types/species (designated

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as RV-A, RV-B, RV-C, RV-D, RV-E, RV-F, RV-G and RV-H) and two new species (RV-I and RV-J) are under consideration of International Committee on Taxonomy of Viruses [4, 13, 26, 27]. The classification is based on antigenicity of the inner capsid VP6 protein as well as the characteristics of genomic RNA [13, 26, 27]. Among all, RV-A is the most prevalent cause of gastroenteritis in humans and animals worldwide [11]. RV-A, B and C are found in both humans and animals, while RV-A, D, F and G are reported in avian species [34, 37] and among that RV-D, F and G are exclusively found in birds [13]. RV-E is detected serologically only in pigs [6] and as per the latest RCWG (Rotavirus Classification Working Group) report, RV-E is considered as a floating group as the virus could not be recovered from the archived samples. Recently, RV-I has been reported from dogs and cats [26, 32] and further the metagenomic analysis of bat fecal samples led to the identification of another group of RV i.e. RV-J [4].

Among the accepted groups of RV (A–H), RV-A is genetically and antigenically the most diverse species [24]. RV-A associated enteritis is a major problem in infants, young calves [33], weaning and post-weaning piglets [17] and foals [29]. RV-A have been identified from other animals like monkeys [3], cats [35], dogs [14], mice [7, 21], rabbits [9] and birds [38]. Due to high genetic variability in the antigenic regions, RV-A are thought to have high interspecies jumping probability which can be coupled with reassortment of cognate genes [23]. To date, 35 different G- and 50 P-genotypes have been described in both humans and animals for RVAs (<https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification>, accessed on 12-12-2017). Twelve G genotypes (G1 to G6, G8 to G12, and G26) and 16 P genotypes (P [13] to P [6], P [33], P [29], P [9], P [22], P [1], P [16], P [8], and P [5]) of RV-A have been associated with pigs [22, 30]. Among them, the predominant G type was found to be G5 which was followed by G3 and G4, respectively and among P type, P [37] > P [34] > P [29] was the order of prevalence in porcine population [31]. Currently, according to RCWG, G and P system for classification of RV has been replaced by a whole genome based classification [25] in which the notation Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (with x indicating the number of the genotype) is used for the VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5 encoding genes, respectively. The number of genotypes recognized by RCWG till date for each gene are 35, 50, 26, 21, 19, 19, 30, 21, 21, 27 and 21 for VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5 encoding genes, respectively.

In India, most of the records on RV epidemiology have originated from north-eastern part of the country where pig farming constitutes major share (40%) of the pig population of the country [1]. Several researchers have identified

the presence of RV-A in pig population of India, but in small sample size. Still, information is limited on the genetic characterization of porcine RV-A isolates from India and as-on-date, only a single whole genome report of porcine RV-A have been analyzed from Indian subcontinent [16]. Here, we describe the prevalence and genetic heterogeneity of RV-A from Indian porcine population.

Materials and methods

Sample collection

Fecal samples (n = 757) were collected from different parts of India including 7 north-eastern states (Assam, Arunachal Pradesh, Meghalaya, Tripura, Mizoram, Manipur, and Nagaland), Uttar Pradesh, Tamil Nadu, Karnataka and Kerala. Diarrheic outbreak and regular diarrhea case samples were collected from organized and unorganized piggeries during the time period of August 2013–December 2017. Sampling was done from piglets of up to 6 months age. The samples collected from each region are given in Table 1.

Sample processing, RNA extraction and first strand cDNA synthesis

A 10% suspension (w/v) of the fecal material was prepared in phosphate buffered saline (PBS; pH 7.2) by dissolving 200 mg of feces in 2 mL of PBS. Clarification of samples was done by thorough mixing of suspended sample on a vortexer and centrifugation at 5000 rpm for 10 min to remove the coarse debris. Total RNA was isolated using Qiazol reagent (Qiagen Sciences, Maryland, USA) following the manufacturer's protocol. Random priming reverse transcription was performed at 37 °C with the extracted RNA (500 ng) by means of Murine Moloney leukemia virus origin reverse transcriptase (MMLV-RT).

RT-PCR based screening of RV-A

For the detection of RV-A, the diagnostic primers (RVA-D-VP6-F 5'-TTTGATCACTAAYTATTCACC-3' and RVA-D-VP6-R 5'-GGTCACATCCTCTCACTA-3') and conditions described in our previous study were used [28]. The thermo cycling conditions used to amplify the 226 bp amplicon include single cycle of initial denaturation at 95 °C for 3 min followed by cyclic denaturation, annealing and extension at 94 °C for 10 s, 50 °C for 10 s and 72 °C for 20 s, respectively. A final extension of 72 °C for 10 min was also given to obtain the finished 226 bp amplicon in RV-A positive samples.

Table 1 Samples screened for porcine RV-A from 11 different states of India

State	Number of samples screened	Porcine RV-A positive	Percent positivity
Assam	236	74	31.35
Arunachal Pradesh	04	03	75.00
Karnataka	21	03	14.20
Kerala	38	15	39.47
Manipur	46	11	23.91
Meghalaya	92	31	34.07
Mizoram	35	08	22.86
Nagaland	43	34	79.07
Tamil Nadu	11	02	18.18
Tripura	79	21	26.58
Uttar Pradesh	152	119	78.29
Total	757	321	42.40

Full length amplification of porcine RV-A capsid VP6 protein gene, cloning and sequencing

A modified primer pair testified by Kerin et al. [20] with approx. 1350 bp product size was used for the full length amplification of the capsid protein (VP6) gene of RV-A. Some of the RV-A positive samples which were not amplifying with full length primer pair were amplified to obtain partial length gene products using custom synthesized primers (TPoRVA F 5'-CAAAAACCTTTGAAAGATGCTAG-3' and TPoRVA R 5'-AATCCAGCTAC TTGAATTTCTGA-3') with an amplicon of approximately 600 bp. PCR conditions optimized for partial length VP6 gene was having a single cycle of initial denaturation at 95 °C for 3 min followed by cyclic denaturation, annealing and extension at 94 °C for 10 s, 55 °C for 20 s and 72 °C for 30 s, respectively. A final extension of 72 °C for 10 min was given for finishing of the amplified product. The full/partial length VP6 gene products were cloned in pDRIVE (Qiagen GmbH, Hilden, Germany) cloning vector, sequenced by Bigdye terminator Sanger sequencing method in ABI 3730xl sequencer (Eurofins Genomic India Ltd, Bangalore, India).

Pairwise sequence identity and phylogenetic analysis of porcine RV-A VP6 gene

Sequence identity calculation was performed using Clustal V program available in the Megalign software of DNASTAR software package. Sequence identities of VP6 gene of RV-A was calculated in between porcine RV-As of Indian origin. Moreover, a sequence identity calculation was performed with the established I types to identify I genotypes of the current study isolates. To elucidate the genetic identity of the RV-A recognized in the current study, phylogenetic tree was prepared in Molecular Evolutionary Genetic Analysis (MEGA) software (v 6.06) [36]. Best fitting model for each gene under study was identified by running the aligned sequences in 'find

the best DNA/protein model' tool provided in MEGA 6. Phylogenetic reconstruction was performed with maximum likelihood method of dendrogram construction and a bootstrap replicate of 1000 was also given to obtain the maximum statistically significant dendrogram. The most suitable model for phylogenetic tree preparation was identified as Tamura-3 parameter with gamma distribution of all the evolutionary sites.

Results and discussion

RT-PCR based rotavirus-A screening

Diarrhea is the major health problem that young pigs are confronted with. It occurs most frequently during two critical periods: (1) during their stay in the farrowing house and (2) shortly after weaning. Rotavirus (RV) is the top priority gastroenteritis pathogen in swine, being responsible for significant economic losses due to increased mortality, treatment costs, and reduced weight gain. Co- and super-infections further worsen the outcome of primary RV-A infections [22]. Among the eight approved species/types of RVs (A-H), RV-A associated enteritis is a major problem in infants, young calves [33], weaning and post-weaning piglets [18] and foals [29].

In the current study, 757 fecal samples were screened by RT-PCR using the self-designed RV-A primer pairs which confirmed the presence of RV-A with observation of expected amplicon size products of 226 bp. Of the 757, 321 samples (42.4%) were found positive for RV-A and out of these positives, highest identification was 119 (37.07%) from Uttar Pradesh, followed by 74 (23.05%), 34 (10.6%), 31 (9.65%), 21 (6.54%), 15 (4.67%), 11 (3.43%), 8 (2.49%), 3 (0.93%), 3 (0.93%) and 2 (0.62%) from Assam, Nagaland, Meghalaya, Tripura, Kerala, Manipur, Mizoram, Arunachal Pradesh, Karnataka and Tamil Nadu, respectively. All the samples in the study and their state-

wise distribution and the corresponding RV-A screening result are comprehended in Table 1. RV-A being the widely studied group of RVs in all animal species, a wide prevalence range has been reported by different researchers. An extensive review on RV-A from 1976 to 2011 by Papp et al. [31] reported an overall 24% prevalence of RV-A among all the virus associated enteric diseases in swine population. A high prevalence of RV has been documented from the United Kingdom where almost 80–89% RV positive diarrheic episodes were identified in 2008–2009 [8]. In an African country (Tanzania), a high RV prevalence of 41.8% was reported. South East Asian countries like Vietnam has also witnessed an incidence of 32% for RV [2]. In India, most of the records on RV epidemiology have originated from north-eastern parts where pig farming constitutes major share (40%) of pig population of the country. The first porcine RV-A prevalence study came out in late 1990's, where 26.9% serum samples were found positive for RV antibodies using sandwich enzyme-linked immunosorbent assay (ELISA) [5]. Kelkar et al. [19] reported a prevalence of 78.8% from North Eastern India whereas antigen detection using RT-PCR by Mondal et al. [28] reported 10.97% identification of RV in pig fecal samples. In this study, the percent positivity for the RV was 42.4% (321/757) which is quite high in comparison to hitherto reports from Indian subcontinent. A very low positivity was observed by Dubal and coworkers (2013) for RV (10.18%) with a maximum of 30% from the North Eastern region followed by 7.4% from the western region, whereas southern and northern region screened were reported negative for RV infection [12]. In our study, northern and north eastern region of India showed higher RV incidence than southern part of India even though the latter was also showing positivity of 14–40%. Notably, a minimal reduction in the RV identification has been noted in the present study in comparison to the report of Das et al. [10] who testified 42–46% of RV detection in the states of Assam and Tripura where our study showed detection of 31.4 and 26.6%, respectively. Even though, a high seroprevalence of 68.75% have been reported in Arunachal Pradesh by Garam et al. [15], this study results showed no positivity in Arunachal Pradesh for RV which were not in consensus with the former findings.

Phylogenetic analysis and pairwise sequence identity analysis of porcine RV-A

In this study, the VP6 gene was amplified and characterized from representative isolates of Indian porcine RV-A. The phylogenogram of porcine RV-A involved 49 sequences retrieved from the GenBank database. All the diverse VP6 genotypes (I1-3, I5 and I14) identified till date from the porcine species were used for making the phylogenogram.

Upon analysis, two major clades were visible in the phylogenogram topology, one of the ancestor diverged to continue as the I1, I5 and I14 genotypes, whereas the second ancestor diverged to make the I2 genotype (Fig. 1). Our study strains were falling into the clusters of I1 and I5. Based on the phylogeny, it is evident that the Indian porcine RV-A have diverse nucleotide sequences, wherein the nucleotide sequence of the isolates from Uttar Pradesh (UP-30 and UP-34) were clustering into the I1 type, and the isolates from North Eastern states (Mizoram and Meghalaya) clustered into both I1 and I5 type. The I5 type Indian porcine RV-A were showing more phylogenetic relatedness towards Chinese RV-A strains (JF781162; KC113249) and earlier reported Indian strain (DQ204741) from Kolkata. It is evident from the phylogenogram that in contrast to the prevalent I genotype (I5 type) of the isolates submitted in the GenBank, most of the isolates of the current study were of I1 type (Fig. 1). In Meghalaya, both types (I1 and I5) of VP6 gene were identified from RV-A, indicating a transition of VP6 genotype. It is noteworthy to mention that all the VP6 genes from nearby country (China) having porous border with the north-eastern states of India was having I5 type of VP6 gene. Before this study, no published report of I type is available from India, whereas from the analysis of GenBank submission of porcine RV-A strains from Kolkata, it was evident that the VP6 gene of earlier submitted sequence were of another type i.e., I2 type. Comprehending all our results along with the earlier isolates reveal that Indian pig population is having RV-A infection with wide variation within the VP6 gene. Percentage identity (using Megalign software) calculation of the VP6 gene sequences from different porcine RV-A revealed 77.1–97.3% identity with the Indian porcine RV-A strains of the current study. A comparative analysis of sequence identity from Indian porcine RV-A supported that they are falling into two different lineages i.e. I1 and I5. Three partial length porcine RV-A sequences of the current study from Mizoram and Meghalaya (NER MIZ-326; MG478492, NER MIZ-332; MG478493 and NER MEG-334; MG478494) showed maximum identity with other reported porcine RV-A I5 sequences (88.4–96.2%) whereas two full length and two partial length porcine RV-A sequences of the current study showed their maximum identity with reported I1 type porcine RV-A from other countries. Tables 2 and 3 represent the pairwise sequence identity within the Indian porcine RV-A and in between other porcine RV-A reported from different parts of the world.

Even though there are reports of detection of porcine RV-A from India, no VP6 gene based lineage/genotype characterization are available for the target gene. Till date, only a single VP6 type (I2) was reported from swine population of India. Present study involved identification of

Fig. 1 Phylogenetic tree of porcine rotavirus-A with other established porcine rotavirus-A I types. Phylogenetic reconstruction was performed in MEGA 6 using Tamura-3+ G parameter. All the isolate names are written in the order of Virus name/accession number/gene name/strain name/year of isolation/country of origin. Abbreviations

like Po = porcine; Bov = bovine; Hu = human; Can = canine are used in along with the virus name for identifying species of the sample origin from which the virus was isolated. Isolates of current study are marked as either solid dots (partial length VP6 gene sequence) or solid squares (full length sequence)

two new type of VP6 gene (I1 and I5) from RV-A and affirms that the circulating virus is consistently changing its genome type. The phylogeny also clearly mention here the circulation of different variants of RV-A which could be used to see the migration/movement of RVA strains world over. Moreover, substantial identification of these new genotypes from north eastern states of India, which share the porous borders, gives an early indication for restriction in movement of livestock to and from the nearby countries.

As a continuation of this study, a wide epidemiological study for different emerging types of RV should be prompted, based on the nucleic acid detection system to get enough basic knowledge to build up a strong control program for the emerging viruses.

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Table 2 Pairwise sequence identity of VP6 gene from different Indian porcine RV-A isolates of the current study

	NER MIZ-326	NER MIZ-332	NER MEG-334	NER MEG-362	NER MEG-363	KX825943 (Por UP-30)	KX825944 (Por UP-34)
NER MIZ-326	^a	95.2	95.5	83.7	83.3	80.1	82.4
NER MIZ-332	95.2	^a	99.6	83.5	83.2	80.6	80.1
NER MEG-334	95.5	99.6	^a	83.9	83.5	80.2	80.6
NER MEG-362	83.7	83.5	83.9	^a	99.5	89.2	88.9
NER MEG-363	83.3	83.2	83.5	99.5	^a	89.2	88.9
KX825943 (Por UP-30)	80.1	80.6	80.2	89.2	89.2	^a	98.9
KX825944 (Por UP-34)	82.4	80.1	80.6	88.9	88.9	98.9	^a

^aIdentity between same sequence**Table 3** Percent identity of Indian porcine RV-A of the current study with different VP6 genotypes of porcine RV-A reported till date

	Established VP6 genotypes identified from pigs		Genotypes identified in current study	
			I1^a	I5^b
I1			88.5–97.3	81–83.9
I2			77.1–80.6	79–80.8
I5			79.4–84.4	88.4–96.2
I14			75.2–80.1	80.5–81.4

Bold characters given in the table indicates the maximum percentage identity for each group of Indian porcine RV-As from the current study

^aIndian porcine RVAs with Accession numbers-(MIZ326 (MG478492); 332 (MG478493) and MEG-334 (MG478494))

^bIndian porcine RVAs with Accession numbers (MEG-362, 363, KX825943-44)

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