ORIGINAL ARTICLE

# Molecular characterization of porcine group A rotavirus to contain piglet diarrhea for productivity enhancement in North East India

Sushmita Das[1] · Pranab Jyoti Das[2] · Pratap Jyoti Handique[1]

**Abstract** Gastrointestinal tract infections with acute diarrhea are indeed a serious cause of concern that held back the swine industry of North East India and among the various etiological agents causing the disease, group A rotavirus is the most common. In this regard, molecular characterization of the viral strains circulating in the region is of utmost importance for a strategic approach to control the disease. Thus, in the present study, diarrheic fecal samples from piglets of Tripura and Assam were evaluated for genotypic characterization of VP7 and VP4 gene of the virus. The samples positive for group A rotavirus were subjected to G and P type determination by nested- multiplex RT PCR as well as nucleotide sequencing of VP7 and VP4 gene. Nested- multiplex RT PCR revealed the presence of porcine group A rotavirus in the genotypic combination of G1P[6], G3P[6], G8P[6] and G4P[6] in the two states of North East India. Nucleotide sequencing of VP7 and VP4 genes however, revealed emergence of an unusual genotypic combination G26P[13]. VP7 and VP4 nucleotide and its deduced amino acid sequences when compared with global isolates, showed that the present strains shared closest similarity to the porcine rotavirus strain TJ4-1from Japan and hp140 from Eastern India. Identification of a novel genotype G26P[13] in the RVA infected pig population of Tripura and Assam, suggested presence of a greater diversity in rotavirus strains in the region. Thus, it evokes the need for continuous monitoring to assess and consider the strain variability in the design of a suitable vaccine candidate against the virus.

## Introduction

North eastern region of India in which the state of Assam alone possesses 2.10 million pigs which is highest for any state in the country having a total population of 9.06 million (2019 livestock census). Seventy percent of people in India depend on agriculture and livestock for their livelihood. Pigs accounts for about 50 percent of total household income in the remote areas of the northeast region [1] contributing significantly in meat production. Although pigs are the most preferred livestock species in north eastern region amongst the tribal and rural populations, these animals are reared mostly under a low-input production system with improper housing, feeding and management exposing the animals against rotavirus and other pathogens causing acute watery diarrhea which is a proximal cause of mortality in pre-weaning piglets [2, 3] thereby causing great loss in pork production.

Rotaviruses comes under the family *Reoviridae* and the genus rotavirus (RV). It is a non-enveloped virus with icosahedral symmetry which comprise of a segmented dsRNA genome [4] encoded with six structural proteins (VP1, VP2, VP3, VP4, VP6 and VP7) that form the capsid of the virus and six non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6) essential for the viral replication cycle. Based on the serological reactivity and

✉ Pratap Jyoti Handique
drpjh@rediffmail.com

1 Gauhati University, Guwahati, Assam 781014, India

2 National Research Centre On Pig, Rani, Guwahati, Assam 781131, India

genetic variability of VP6, RVs are differentiated into eight different groups, designated as RVA-RVH [5] and two new species (RV-I and RV-J) are recently proposed by International Committee on Taxonomy of Viruses (https://talk.ictvonline.org/taxonomy/). RVAs are classified into G (for glycoprotein) and P (for protease-sensitive) genotypes based on VP7 and VP4 genes [6, 7]. VP7 and VP4 genes of RVA elicit neutralizing antibodies and hence, information on the diversity of these genes is significant for vaccine development [5]. Currently, 36 G and 51 P types were described for RVA [https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification/rcwg]. From global epidemiological surveys, 12 different G-types (G1 to G6, G8 to G12 and G26) and 13 different P-types (P[1], P[5] to P[8], P[11], P[13], P[19], P[23], P[26], P[27], P[32], P[34]) have been identified and reported to be associated with piglet diarrhea caused by RVA [8–10]. In India, 6 types of G (G3, G4, G6, G9, G10, G12) and 5 types of P (P[6], P[7], P[8], P[13], P[19]) of RVA have been detected from pigs so far (11–13). Due to the segmented genome of RV, there is an increase diversification taking place in RV strains [13]. Thus, it evokes the need for continuous monitoring to assess and consider the strain variability in the design of a suitable vaccine candidate.

Reports on molecular characterization of porcine RVA are very scanty from North East (NE) India, although, the presence of RV associated piglet diarrhea has been documented [11, 14]. As such, there is an urgent need not only to study the incidence of RV infection in pigs, but also the genotype determination and molecular characterization of the viral strains that are circulating in the region. Thus, the present study was designed to investigate the RVA strains circulating in the states of Assam and Tripura of NE India with a view to have some baseline information to formulate future vaccine strategy for safeguarding the swine industry from this important pathogen. The investigation also emphasized to report presence of any new RVA strain, if circulating in the region.

## Materials and methods

### Viral sample

A total of three hundred twenty diarrheic fecal samples were collected from loose housing and farm borne piglets in the age group of 0–6 months from Assam and Tripura states of NE India. The fecal samples were collected in sterile scintillation vials (Tarsons, India) and transported on ice to laboratory for further processing. Fecal sample brought on ice was thawed under room temperature and approximately, 1 g of the sample was suspended in four volumes of 0.06 M PBS (pH = 7.2). The suspension was then homogenized and were thereafter centrifuged at 12,000xg at 4 $^\circ$C. The supernatant of every sample was collected in sterile 2 ML Eppendorf vials (Eppendorf, Germany) as 20% fecal suspension. The processed samples were preserved at −20 $^\circ$C for further use.

### RNA extraction and amplification of VP7 and VP4 gene

Initial screening of RV in processed fecal samples were detected by using a solid phase sandwich type enzyme immunoassay (Premier RotacloneTM, Meridian Biosciences, United States) as per the manufacturer's protocol. Viral RNA of the ELISA positive samples was extracted by QIAamp viral RNA mini kit (QIAGEN, Germany) and was checked for purity and concentration using nanodrop spectrophotometer (Thermo Fisher Scientific, United States). Viral RNA samples lying in the ranges of OD ratio (260:280) between 1.80–2.00 were subjected to amplification of VP7 (1062 bp) and VP4 (876 bp) genes [15, 16].

### G and P genotyping of porcine RVA by nested-multiplex RT PCR

Full length VP7 amplicons (1062 bp) were used as template for G typing which were obtained from the first round of amplification of VP7 gene by RT PCR. Six genotyping primers (aAT8, aBT1, aCT2, aDT4, aET3, aFT9) along with the VP7 consensus primer RVG9 [15] were used in combination for determination of the prevalent G types in the RVA positive samples. On the other hand, partial VP4 amplicons generated from the first round of amplification by RT PCR were used as template for P typing. The P genotypes were determined by nested-multiplex RT PCR by using a cocktail of five minus-sense type specific primers (1 T-1, 2 T-1, 3 T-1, 4 T-1, 5 T-1) and the plus-sense consensus primer Con3 [16]. The PCR products (G and P types) so generated were analyzed on 1.2% agarose gel against a 100 bp DNA ladder (Thermo Fisher Scientific, United States).

### Sequencing and phylogenetic analysis of VP7 and VP4 gene

The non- typeable VP7 and VP4 amplicons by nested-multiplex RT PCR, were purified using QIAquick PCR purification kit (QIAGEN, Germany) and was sequenced at Merck Specialities Private Limited, Bangalore, Karnataka, India. Nucleotide sequences were determined using an automated ABI 3100 Genetic Analyzer V.5.1. Sequenced VP7 and VP4 genes of porcine RVA were submitted to NCBI GenBank database and the sequences were analyzed by erstwhile RotaC v2.0 (http://rotac.regatools.be. which is

now functional on ViPRBRC website, https://www.viprbrc.org/brc/rvaGenotyper.spg?method=ShowCleanInputPage&decorator=reo), a web-based tool for the genotyping analyses of the RV sequences in accordance with the Rotavirus Classification Working Group (RCWG) [17]. Further, the nucleotide and deduced amino acid sequences of VP7 and VP4 genes were compared with other global isolates of porcine, human and bovine available at NCBI GenBank database using the Blast server. For phylogenetic study, the sequences obtained were aligned using the ClustalW program [18] and the alignment was corrected manually. To estimate the genetic divergence of the porcine RVA strains with regard to VP7 and VP4 gene, both intra- and inter-phylogenetic distances were calculated using the Kimura-2-parameter (K2P) model by MEGA v.6 [19]. Distance metrics between the present isolates with that of the reference strains selected on the basis of type species nucleotide sequence for porcine RVA, retrieved from the NCBI GenBank database were calculated with MegAlign software of DNASTAR software package [18]. Mismatch analysis values were estimated using 100% bootstraps value and these values were used to construct the phylogenetic tree using the neighbor- joining method [20].

## Results and discussion

### Detection of porcine RVA

There is a wide distribution of rotavirus associated piglet diarrhea in different parts of India [2, 3, 21, 22] with reports of higher occurrence in the north eastern states [12, 14, 22, 23]. In the present study, out of 320 samples (120 from Assam and 200 from Tripura) screened by Monoclonal Antibody based sELISA (Premier Rota-cloneTM, Meridian Biosciences, United States) 130 samples (40.62%) were detected to be positive for RVA with an OD measure which ranged between 0.150 and 0.351. The positive cases in Assam were found to be 47/120 (39.16%) while, in Tripura it was 83/200 (41.50%) for RVA. The ELISA positive samples showed successful amplification of both VP7 and VP4 region of porcine RV by RT PCR. Notably, RVA identification from the present findings is higher in the two states in comparison to a recent report where Assam and Tripura had a positivity percentage of 23.05% and 6.54% respectively [23].

### Distribution of G and P genotypes

In addition to the most prevalent RVA strains circulating worldwide, several rare and/or novel G (G1, G2-like, G6, G8, G9, G10, and G12) and P types (P[1], P[5], P[8], P[11], P[13], P[19], P14[23], P[26]) have been detected

sporadically in pigs [13]. The high incidence of diversity in the RV strains is predominantly due to accumulation of point mutations leading to genetic/antigenic drift and reassortment of cognate genes leading to genetic/ antigenic shift. In view of the zoonotic and public health importance of RV infection and for development of efficacious vaccines for protection of pig populations of the region, epidemiological reports related to the prevalence and genotype distribution (G and P types) of porcine RVA is of utmost importance [24]. Thus, documentation of the emerging porcine RV strains circulating in the north eastern region of India is essentially needed.

In the present study, three different G types: G1 (749 bp), G3 (374 bp) and G8 (885 bp) were detected from the samples of Assam. Of these, G3 was the most prevalent genotype (20/47; 42.55%), followed by G8 (13/47; 27.66%) and G1 (10/47; 21.27%). Mixed infection with more than one G types was also evident, which was G1-G4 (3/47; 6.38%). On the other hand, only one G type: G4 (583 bp) was confirmed among the samples from Tripura (78/83, 93.97%). These results are evident from the agarose gel photographs (Fig. 1 a, b). On the contrary, a single P type: P[6] was identified from the two states of NE India (Fig. 1 c). Thus, the most common G-P combinations seen in the samples from Assam were: G3P[6], G8P[6], G1P[6] and G1-G4P[6] while, samples from Tripura revealed only one pattern of G-P combination as G4P[6].

A sequence based study from Assam reported the presence of major G and [P] combination to be G1P[7], G1P[13] and G1P[23] from porcine infected with RVA [25]. Another genotypic characterization study from Assam and Arunachal Pradesh of north east India revealed distribution of G3P[6], G3P[7], G9P[6], G9P[7], G10P[7], G10P[8] and G9P[8] [11, 12]. However, from the present study, the distribution of G and P types came out to be G1, G3 and G8 from Assam, G4 type from Tripura and a single P[6] type from both the states. Thus, molecular typing of porcine RVA from the pig population of Assam revealed that G1, G3 and P[6] strains are circulating continuously as evident from our investigation with that of the earlier reports. The current findings also revealed a mixed infection with more than one G type (G1-G4) from Assam and such co-infection of two different RV strains (G1 and G4) in the same host may lead to generation of reassortants due to exchange of gene segments.

### Sequence and phylogenetic analysis of VP7 and VP4 gene

The accession numbers retrieved from NCBI GenBank database against the non-typeable VP7 and VP4 gene sequences of porcine RVA were: KT277523, KT277524, KT277525, KT277526, KT277527 and KT338642,
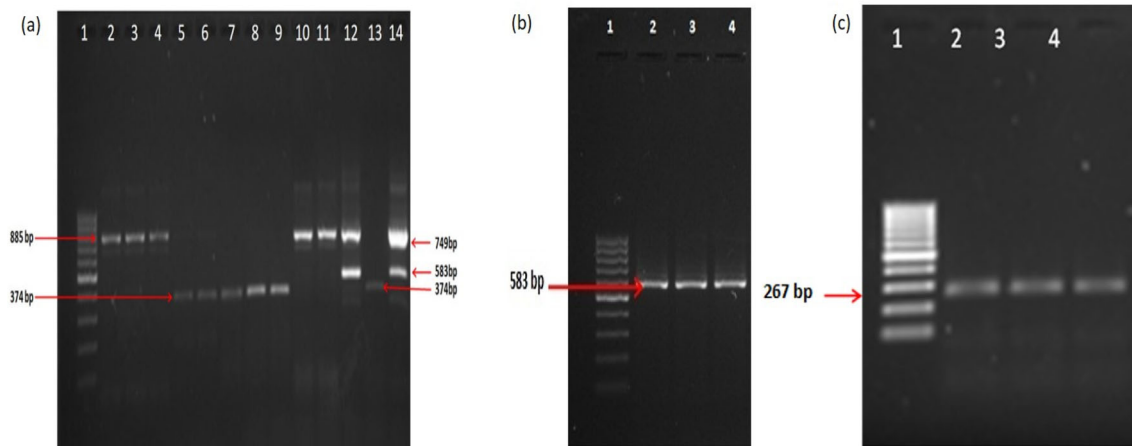
**Fig. 1** G and P typing of porcine RVA by nested-multiplex RT PCR. **a.** Samples from Assam; Lane 1: DNA ladder (100 bp), Lane 2–4: G8 type (885 bp), Lane 5–9, 13: G3 type (374 bp), Lane 10–11: G1 type (749 bp), Lane 12, 14: mixed G types G1 (749 bp)-G4 (583 bp). **b.** Samples from Tripura; Lane 1: DNA ladder (100 bp), Lane 2–4: G4 type (583 bp). **c** Samples from Assam and Tripura; Lane 1: DNA ladder (100 bp), Lane 2–4: P [6] type (267 bp)

KT338643, KT338644, KT338645, KT338646 for Tripura strains and KT277528 and KT338647, KT338648 for Assam strains.

RotaC confirmed that all the VP7 and VP4 nucleotide sequences from Tripura and Assam belong to porcine RVA and shares closest sequence similarity with that of G26-RVA/Pig-wt/JPN/TJ4-1/2010/G26Px and P13-RVA/Pig-tc/XXX/A46/XXX/G5P13 respectively. Thus, all the present isolates were assigned as genotype G26P[13] as their percentage identity cut-off value is higher than 80%. The exact relationship between the VP7 and VP4 genes of the present isolates with that of the cognate genes of all established genotypes have been represented in the phylogenetic tree so generated by RotaC software (Online Resource 1, Online Resource 2). In the present study, emergence of an unusual porcine RVA genotypic combination of G26P[13] was found to be circulating between the two states of NE India.

Comparing the full-length nucleotide and deduced amino acid sequences of VP7 gene of the present isolates (KT277523- KT277528) with the reference strains from GenBank revealed that all the present strains shared closest similarity to the porcine RV strain TJ4-1(AB605258, BAJ46511) from Japan both at the nucleotide level (86.0-94.5%) as shown in Fig. 2 (a) and at the amino acid level (91.7–96.9%) as in Fig. 2 (b). The phylogenetic tree (Fig. 2c) revealed that the present strains clustered together with the TJ4-1 strain (AB605258) of G26 genotype. With regard to the two outgroups considered for the study, the phylogenetic cluster of the present porcine RV strains is closer to its bovine counterpart than human.

Comparison among the partial VP4 nucleotide and its putative amino acid sequences of the isolates of the study with the reference strains of porcine, bovine and human origin from GenBank revealed that all the present isolates (KT338642-KT338648) shared high identity with a porcine strain hp140 (AY818362, AAX13501) from eastern India both in terms of nucleotide (87.8–89.1%) as well as in terms of amino acid (88.3–91.0%) as shown in Fig. 3 (a, b). Further, it is evident from the phylogenetic tree (Fig. 3c) that all the isolates clustered together with the strain hp140 (AY818362). However, with respect to the two outgroups, VP4 gene of the present porcine isolates is closer with its human counterpart than bovine.

The present study therefore, revealed emergence of a novel porcine RVA genotypic combination of G26P[13] circulating between the two states of the north eastern region of India. The genotype P[13] of the present study showed homology to strain hp140 isolated from pig farms located in the eastern state of West Bengal [26]. However, detection of G26 genotype homologous to porcine strain TJ4-1 of Japan found to be present in the north eastern region of India has incited to focus on the need of continuous surveillance and its molecular characterization. Besides, the emergence of an unusual genotype also evoked the need of whole genome sequence studies in order to continuously monitor the distribution of RVA strains. The results of the present investigation will therefore aid in development of an efficacious candidate vaccine strain for safeguarding the porcine industry and reducing the major impediment in productivity enhancement of the pig populations.

Thus, molecular characterization of porcine RVA from two states of north eastern region of India for the first time revealed occurrence of some noteworthy events of the virus which included co-infection with two strains in the same host, emergence of a new genotypic combination of G with P types (G26P[13]), and phylogenetic cluster revealing the
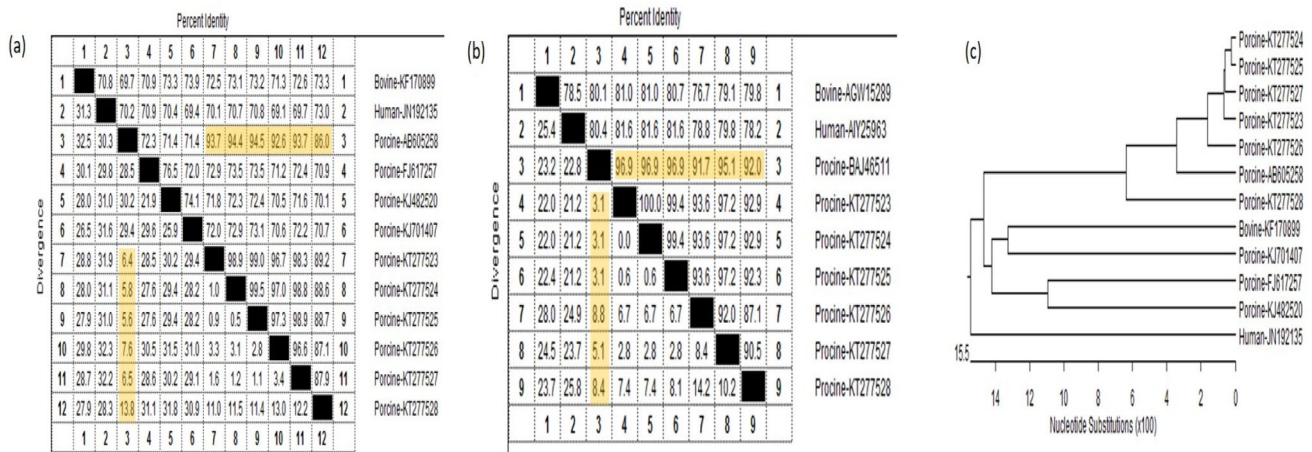
**Fig. 2 a, b.** Pair distances matrix of VP7 gene and its deduced amino acid sequence of porcine RVA with porcine, human and bovine sequences showing percent identity and divergence with its closest porcine strain TJ4-1 (AB605258, BJ46511). **(c).** Phylogenetic tree constructed using bootstrap value comparing sequence similarity of VP7 gene of porcine RVA with its closest porcine, human and bovine sequences. The resultant phylogenetic tree shows the branch length 15.5 proportional to the number of substitutions per site
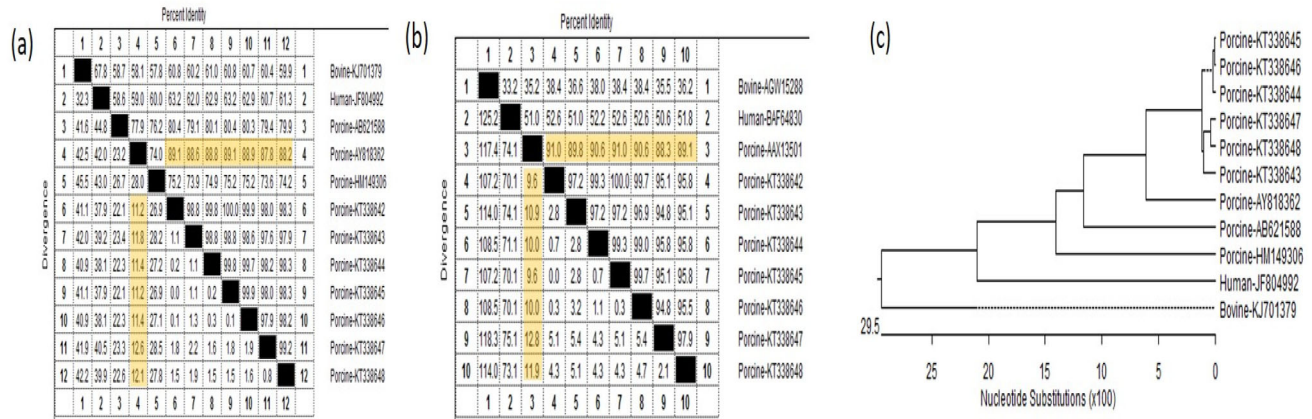


**Fig. 3 a, b.** Pair distances matrix of VP4 gene and its deduced amino acid sequence of porcine RVA with porcine, human and bovine sequences showing percent identity and divergence with its closest porcine strain hp140 (AY818362, AAX13501). **c.** Phylogenetic tree constructed using bootstrap value comparing sequence similarity of VP4 gene of porcine RVA with its closest porcine, human and bovine sequences. The resultant phylogenetic tree shows the branch length 29.5 proportional to the number of substitutions per site

lineage relationship with other host species. The findings of the present investigation in conjunction with an earlier report [22] suggest a wide epidemiological study for different emerging types of RV based on molecular detection techniques in order to develop an efficient control program for the emerging viruses. Moreover, the current findings emphasized need of continuous surveillance to create an immune belt across the regional borders of the country to protect pig health, thereby, raising household economy of the rural and tribal farmers.

**Data Availability** The GenBank accession numbers for VP7 and VP4 genes of RVA are KT277523 to KT277528 and KT338642 to KT338648 respectively.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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