Virology 598 (2024) 110195

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Complete genome constellation of a dominant Bovine rotavirus genotype circulating in Bangladesh reveals NSP4 intragenic recombination with human strains

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ARTICLE INFO

Handling Editor: Dr. Jasmine Tomar

Keywords:
Bovine rotavirus A
NGS
Whole genome constellation
DS-1
G6P[11]
NSP4

ABSTRACT

Rotavirus A is a leading cause of non-bacterial gastroenteritis in humans and domesticated animals. Despite the vast diversity of bovine Rotavirus A strains documented in South Asian countries, there are very few whole genomes available for phylogenetic study. A cross-sectional study identified a high prevalence of the G6P[11] genotype of bovine Rotavirus A circulating in the commercial cattle population in Bangladesh. Next-generation sequencing and downstream phylogenetic analysis unveiled all 11 complete gene segments of this strain (BD_ROTA_CVASU), classifying it under the genomic constellation G6P[11]-I2-R2-C2-M2-A13-N2-T6-E2-H3, which belongs to a classical DS-1-like genomic backbone. We found strong evidence of intragenic recombination between human and bovine strains in the Non-structural protein 4 (NSP4) gene, which encodes a multifunctional enterotoxin. Our analyses highlight frequent zoonotic transmissions of rotaviruses in diverse human-animal interfaces, which might have contributed to the evolution and pathogenesis of this dominant genotype circulating in the commercial cattle population in Bangladesh.

1. Introduction

Rotavirus A (RVA) is a genetically and antigenically diverse species in the genus *Rotavirus,* Family *Sedoreoviridae* and is considered the leading cause of viral gastroenteritis in humans and animals worldwide (Martella et al., 2010). After being first reported almost four decades ago, numerous RVA strains and genotypes have been detected in cattle populations and thereafter anecdotally classified as bovine Rotavirus A (BRV) in the scientific literatures (Ghosh et al., 2007; Hassine-Zaafrane et al., 2014; Mebus et al., 1971; Pisanelli et al., 2005; Tatte et al., 2019). BRVs are associated with high morbidity and mortality in newborn calves and renders significant negative impact on the cattle industry throughout the world (Cho and Yoon, 2014).

The genome of RVA consists of 11 segments of double-stranded RNA enclosed in a triple-layered virus particle, and encodes for six structural (VP1–VP4, VP6, and VP7) and five or six non-structural proteins (NSP1–NSP6) (Hu et al., 2012). Classification of RVA is based on neutralising antibodies against the capsid proteins VP7 (G; glycoprotein) and VP4 (P; protease-sensitive protein) which identified at least 42 G and 58 P genotypes across the world (RCWG, 2021; Le et al., 2024). This dual genotyping method has resulted in a identification of large diversity of RVA stains in epidemiological studies among which the combinations of G9P[8], G1P[8], G2P[4], G3P[8], G4P[8] and G12P[8] are historically considered the most prevalent in human population worldwide (Bányai et al., 2018; Wahyuni et al., 2021). However in cattle, at least 12 different G genotypes and 11 different P genotypes

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https://doi.org/10.1016/j.virol.2024.110195
Received 13 June 2024; Received in revised form 22 July 2024; Accepted 28 July 2024
Available online 30 July 2024

have been reported to date where G6, G8, G10 combined with P[1], P[5] and P[11], respectively are the most common genotypes (Hassine-Zaafrane et al., 2014; Matthijnssens et al., 2008; Tatte et al., 2019). Given the segmented nature of the rotavirus genome, genes encoding each segment can undergo independent reassortment *in-vivo* following co-infection with different rotavirus strains (Dennehy, 2008; Maunula and Von Bonsdorff, 2002; Ramig, 1997). Reassortment (Estes and Greenberg, 2013; Ramig, 1997) and recombination (Woods, 2015) are clearly the major drivers for the genetic diversity, evolution and adaptation of rotaviruses (Ghosh and Kobayashi, 2011). Whole genome sequencing and analyses of the genome constellation has become the method of choice (Matthijnssens et al., 2012) for investigating those mechanisms. A whole genome-based genotyping system was developed for RVAs with the aim of facilitating tracing of the origins and evolutionary dynamics of RVAs (Matthijnssens et al., 2008) that included all 11 genome segments of RVA (Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx) where x indicating the numbers of the corresponding genotypes (Matthijnssens et al., 2011). Such classification allowed the vast diversity of genotype segments (32 G, 47 P, 24 I, 18 R, 17 C, 17 M, 28 A, 18 N, 19 T, 24 E, and 19 H) into three major genogroup constellations. The majority of human RVA strains possess either the Wa-like genogroup 1 constellation (Gx-P[x]-I1-R1-C1-M1-A1-N1-T1-E1-H1), which contains mostly strains of porcine origin, or the DS-1-like genogroup 2 constellation (Gx-P[x]-I2-R2-C2-M2-A2-N2-T2-E2-H2), which consists of strains typically of bovine origin. Occasionally, human RVA strains have the AU-1-like genogroup 3 constellation (Gx-P[x]-I3-R3-C3-M3-A3-N3--T3-E3-H3) that comprised strains mostly of feline origin (Estes and Greenberg, 2013; Matthijnssens et al., 2008).

A growing number of reports demonstrated inter-species transmission of RVA among animals and from animals to humans (Gautam et al., 2015; Medici et al., 2015; Zhou et al., 2015). Such zoonotic rotaviruses can become increasingly "humanized" by reassortment with co-infecting human viruses (Matthijnssens et al., 2010b; Theuns et al., 2015). For instance, the majority of these bovine-like rotaviruses recovered from zoonotic infections in humans were inter genogroup reassortant rotaviruses where the nucleotide sequence of some of the segments were highly related to the sequences of cognate segments of bovine rotaviruses (Gollop et al., 1998; Palombo and Bishop, 1995). However, occasional clinical disease from bovine rotavirus infections with DS-1 genomic backbone was reported in Israel (Doan et al., 2013). At the same time, intragenic recombination also playing a significant role in evolution, diversity as well as pathogenicity of RVAs (Hoxie and Dennehy, 2020). As a prevalent genotype, G6P[11] rotavirus in cattle has been repeatedly reported, but there is paucity in the evidence of intragenic recombination in the NSP4 gene of G6P[11] bovine rotavirus with human strain.

In Bangladesh, rapid urbanization and high density of human–livestock interface may pose a positive multiplier effect on zoonotic transmission of RVA of bovine origin. Many RVA stains are highly prevalent in Bangladesh resulting 6000–14,000 deaths each year, particularly among children of less than 5 years of old (Tanaka et al., 2007). Outer Capsid Glycoprotein (VP7) coding genotype G2 has been identified as the most common genotype of human rotaviruses (HRVs) circulating for the past 20 years, while G9 and G12 emerged as predominant genotypes in the last decade (Afrad et al., 2014; Dey et al., 2009; Paul et al., 2008). One study documented high prevalence of typical DS-1-like constellation among HRV strains where G2P[4] was the most common genotype persistently prevalent in South Asia including Bangladesh (Aida et al., 2016). In contrast, a recent study identified high prevalence of G8 followed by G10 and G6 genotypes within cattle and goat population of Bangladesh (Hossain et al., 2020). The most common VP7/VP4 combinations for cattle are G10P[11], G10P[15], and G6P[11], and for goat, G8P[1], and G10P[1] (Hossain et al., 2020).

Whole genomes have been characterised for several Bangladesh human RVA strains including G1, G2, G6 and G12 (Afrad et al., 2013; Aida et al., 2016; Ghosh et al., 2011; Rahman et al., 2010). A paucity of genomic characterisation for bovine *Rotavirus A* strains circulating in Bangladesh limits a full understanding of the potential role of spillover infections between humans and cattle. Likewise, no studies have incorporated global recombination analysis to assess genealogical relationship among diverse RVA strains which might provide critical insight into diversity and transmission. Here we characterised the complete genome constellation of genotype G6P [11], the predominant strain circulating in cattle population of Bangladesh. We also report statistically significant recombination breakpoints within a virulent gene of this strain, highlighting the importance of genetic surveillance in scenarios where anthropozoonotic transmission can be a potential driver for extant genetic diversity.

2. Materials and methods

2.1. Sampling, molecular detection and genotype screening of BRA

A total of 411 faecal contents were collected randomly from diarrheic and non-diarrheic calves in 209 commercial dairy farms of Chattogram division, the south-eastern part of Bangladesh during the period from July 2015 to May 2016. Antigen capture ELISA kit (Bio-X® Diagnostics; Jemelle, Belgium) was used for initial screening of bovine rotavirus (BVR) infection following the manufacturer's instruction. All ELISA positive samples were subjected to viral RNA extraction using a magnetic bead-based system, MagMAX™-96 Viral RNA Isolation Kit (Ambion, Austin, TX, USA) and subsequent reverse transcriptase polymerase chain reaction (RT-PCR) was assayed for targeted amplification of BRV specific outer capsid genes (VP7 and VP4). Prior RT-PCR assay, the double stranded viral RNA was denatured by snap heating at 95 °C for 5 min and immediately chilled in crushed ice. The RT-PCR reactions were performed by using QIAGEN One Step RT-PCR Kit (QIAGEN, USA) for the confirmation of BRA. The primer sets and thermal conditions for RT-PCR assays were set following the protocol described by Iturriza-Gómara et al. (2004) for amplification of partial segments of VP7 gene (891bp) and Rahman et al. (2005) for VP4 gene (861bp). Both amplicons were subjected to agarose gel electrophoresis, stained with ethidium bromide and examined under ultraviolet light for appropriate amplicon size of the respective genes. The amplicons were then purified by PCR purification kit (FavorPrepTM PCR Clean-Up Mini kit, Favorgen, Taiwan) according to the manufacturer's instructions and subsequently sent for Sanger sequencing. All raw sequence data (chromatogram) of partial VP7 and VP4 gene segments were carefully analysed and cleaned up for subsequent rotavirus genotype association screening using a web-based tool, RotaC v2.0 (Final accessed on 18 April 2019) (Maes et al., 2009). The partial sequences were also subjected to NCBI Blast for further confirmation of the genealogical association of respective BRA isolates.

2.2. Next generation sequencing

Since there was only one genotype of BRA was identified on genotypic screening a single strain (BD_Rota_CVASU) was selected for next generation sequencing to obtain the whole genome. The selected fecal sample (100 μL) was suspended in phosphate-buffered saline (900 μL) and vortexed vigorously for 10 min and consequently centrifuged at 13,400 RCF for 10 min to obtain the supernatant. A total 500 μL of supernatant was collected and filtered through a 0.45-μm filter (Millipore) to remove eukaryotic and bacterial cell-sized particles. For further processing, the filtrate was then collected in two 1.5 mL centrifuge tubes, each containing ~200 μL of the filtered suspension and divided into two sample preps (first and second). These two sample preps were then subjected to either direct RNA extraction or nuclease treatment. The first sample was subjected to total RNA extraction using QIAGEN RNeasy Mini Kit (QIAGEN, USA) as per manufacturer's instruction. On the other hand, the second set underwent nuclease treatment for preferential enrichment of viral RNA (Yang et al., 2016; Zhang et al., 2016).

In short, the filtrate was treated with DNase (Turbo DNase from Ambion, Baseline-ZERO from Epicentre, and Benzonase from Novagen) and RNase (Fermentas) at 37 °C for 60 min to digest unprotected nucleic acid to reduce levels of bovine nucleic acids, while viral genomes remain protected within the capsid shell (Yang et al., 2016). Subsequently this nuclease treated sample was subjected to total RNA extraction similar to the first sample prep.

Viral cDNA synthesis was conducted using the 10 μL extracted viral RNA template (from both prep) using 100 pmol of random hexamer (IDT) at 72 °C for 2 min followed by adding 200U SuperScript III reverse transcriptase (Invitrogen), 0.5 mM of each deoxynuceloside triphosphate (dNTP), 10 mM dithiothreitol, and 1 × first-strand extension buffer to the mixture and incubated at 25 °C for 10 min followed by 50 °C incubation for 1 h. Reverse transcriptase was then inactivated by incubating in 70 °C for 15 min. The second strand of cDNA synthesis was performed by incubation of reverse transcribed (RT) products with 5U of Klenow Fragment DNA polymerase (New England Biolabs) at 37 °C for 1 h followed by 75 °C for 20 min. The resulting double stranded cDNA from both sample preps was used as input template for Nextera XT DNA library construction using manufacturers protocol (Illumina) and sequenced using the MiSeq Illumina platform to obtain 300 base paired ends with a distinct molecular tag (adapter) for each set of sample prep.

2.3. Metagenomic screening

The raw reads (FastQ) obtained from both untreated and enriched sample sets were screened using a cloud-computing pipeline, MePIC (Takeuchi et al., 2014) to detect and compare viral signals in each sample sets. In this pipeline the sequence reads were matched against a database of known sequences, and the unnecessary bases were reduced by subtracting reads derived from host genome (*Bos taurus*). Subsequently, an interactive metagenomics visualization web tool (Krona) was used to intuitive exploration of relative abundances of pathogens within the complex hierarchies of metagenomic classifications (Ondov et al., 2011).

2.4. Genome assembly, segment sorting and annotation

A total of 871,878 and 1,085,186 raw 301-bp reads were obtained from the sample untreated and nuclease treated sample set, respectively. Preliminary quality evaluation for all raw reads was generated, pre-processed to remove ambiguous base calls and poor-quality reads, and adapter sequence was trimmed using BBDuk (https://www.geneious.com/plugins/bbduk/). De novo assembly was performed using Geneious and Tadpole assembler implemented in Geneious v 11.1 software package. This approach generated 17,870 and 5181 Tadpole contigs while Geneious assembler produced 2270 and 1750 contigs. A local BLAST pipeline was created in Geneious v11.1 using the genome constellation of Bovine Rotavirus A strain as reference and a low complexity Megablast with default parameter (Mismatch scoring 1–2, Max E-value 10 with open extend linear Gap cost) was used to identify, flag and bin de novo assembly contigs. Subsequently, the highest quality contigs were mapped separately to reference rotavirus segments using both medium and high custom sensitivity parameters where sequence reads with more than 80% overlap identity were used to build the consensus sequence of the eleven (11) rotavirus genome segments. For further confirmation the draft segments were mapped against the raw dataset using Geneious Reference mapper and BBMap (https://sourceforge.net/projects/bbmap) followed by gap tweaked, ordered and oriented manually where necessary. The final genome segments thus generated matched perfectly with the results from de novo assembly. Afterwards, Glimmer V. 3.0 (Delcher et al., 2007), was used to annotate each gene from the final assembled segments. RotaC version 2 (http://rotac.regatools.be/), a classification tool for RVAs, was used to assign genotypes to all eleven genome segments. Nucleotide sequences generated in this study were deposited into the NCBI GenBank.

2.5. Phylogenetic reconstruction

To ascertain the evolutionary relationship of the newly assembled Bovine rotavirus A segments we compiled publicly available full-length rotavirus segments from NCBI Virus variation database (rotavirus resources) for phylogenetic reconstruction. Since our research was focused on domestic animal and human interface in south Asian region the sequence dataset was compiled accordingly. Individual sequences were annotated with accession number, host and segment name. A global alignment of all full-length genome segment were generated in Geneious with MAFFT v 7.017 using G-INS-i (gap open penalty 1.53; off set value 0.123) alignment algorithm (Katoh et al., 2002). The programme jModelTest 2.1.3 favoured a general-time-reversible substitution model with gamma distribution rate variation and a proportion of invariable sites (GTR + I + G4) were estimated using the program PhyML v3.1 (Guindon et al., 2010). Branch support was evaluated by bootstrap analysis based on 1000 pseudo-replicates. FigTree v1.4 was used to generate the consensus trees of different segment.

2.6. Comparative genomics

The relative closeness of the newly assembled rotavirus genome was compared and alignment results were generated and visualized using mVISTA (Frazer et al., 2004) (http://genome.lbl.gov/vista/mvista/submit.shtml; Accessed on April 17, 2019) with closely related genome constellations identified in domestic animal and human population.

2.7. Recombination analysis

To explore whether any intragenic recombination has contributed in the evolution of the genome constellation all complete rotavirus genomes from NCBI's Virus Variation Resource as of January 2023 (n = 61,920; Supplementary table-1) were downloaded (Hatcher et al., 2017). Strains from human, mammal, bird, primate and shellfish were included in recombination analyses. To cover each rotavirus genome, separate fasta files were downloaded for each of the eleven segments. All eleven segments of each complete genome were separately aligned using MAFFT v6.864 (Katoh et al., 2005) after removing any low quality sequence (e.g., 'Ns'). Putative recombinants were identified using a stable RDP4 Beta 4.10, a program, which employs multiple recombination detection methods to minimize the possibility of false positives (Martin et al., 2015). All genomes were analysed, with a p-value cut-off $<10^{-4}$, using 3Seq, Chimaera, SiScan, MaxChi, Bootscan, Geneconv, and RDP methods as implemented in RDP4 (Martin et al., 2015). A recombinant event was considered conceivable with significant p-values (<0.05) as well as detected by at least three of the above-mentioned methods. Sequences in the analysed dataset that most closely resembled the parental sequences of recombinants were defined as either "minor parents" or "major parents" based on the size of the genome fragments (the major parent contributing the larger fragment and the minor parent the smaller) that these sequences had contributed to the detected recombinants.

3. Results

3.1. Molecular identification and genotype screening

A total of twenty-one samples (16 from diarrheic and 5 from non-diarrheic samples) were positive for RVA antigen in both by ELISA and RT-PCR assay targeting VP7 and VP4 genes. The amplicons were further verified using sanger sequencing (data not shown) and analysed for genotype association using RotaC which revealed that all samples belong to a single genotype G6P[11] and therefore indexed under the strain BRA/Calf-Wt/Bangladesh/CVASU/2016/G6P[11].

3.2. Metagenomic screening

Viral signals were significantly increased after RNA enrichment (68% of root) than fecal samples (7% of root). RNA enriched sample revealed 85% of viral signals where 100% covered rotavirus. Simultaneously, several *Enterobacteriaceae* family members, Gram-positive and Gram-negative organisms were also detected within the complex hierarchies of metagenomic classifications (Supplementary Fig. 1). After RNA enrichment, signal of *Olsenella umbonate,* a variably Gram-positive, anaerobic and non-spore-forming bacterium was significantly reduced (Supplementary Fig. 1).

3.3. Whole genome constellation of strain BD_ROTA_CVASU

The full length open reading frames of the 11 genome segments of strain BRA/CalfWt/Bangladesh/CVASU/2016/G6P[11] were successfully determined using illumina MiSeq technology and deposited in GenBank under accession numbers MK376887-MK376891 for NSP1-NSP5 genes and MK376892-MK376897 for VP1-VP4, VP6 and VP7 genes respectively. The closest strains of each genome segment were determined based on default megablast parameters implemented in NCBI BLAST search as of April 2020 revealing highest pairwise nucleotide identity of different rotavirus strains from both human and animal hosts (Supplementary Table 2). The whole genome constellation was determined to be G6-P[11]-I2-R2-C2-M2-A13-N2-T6-E2-H3 which is a

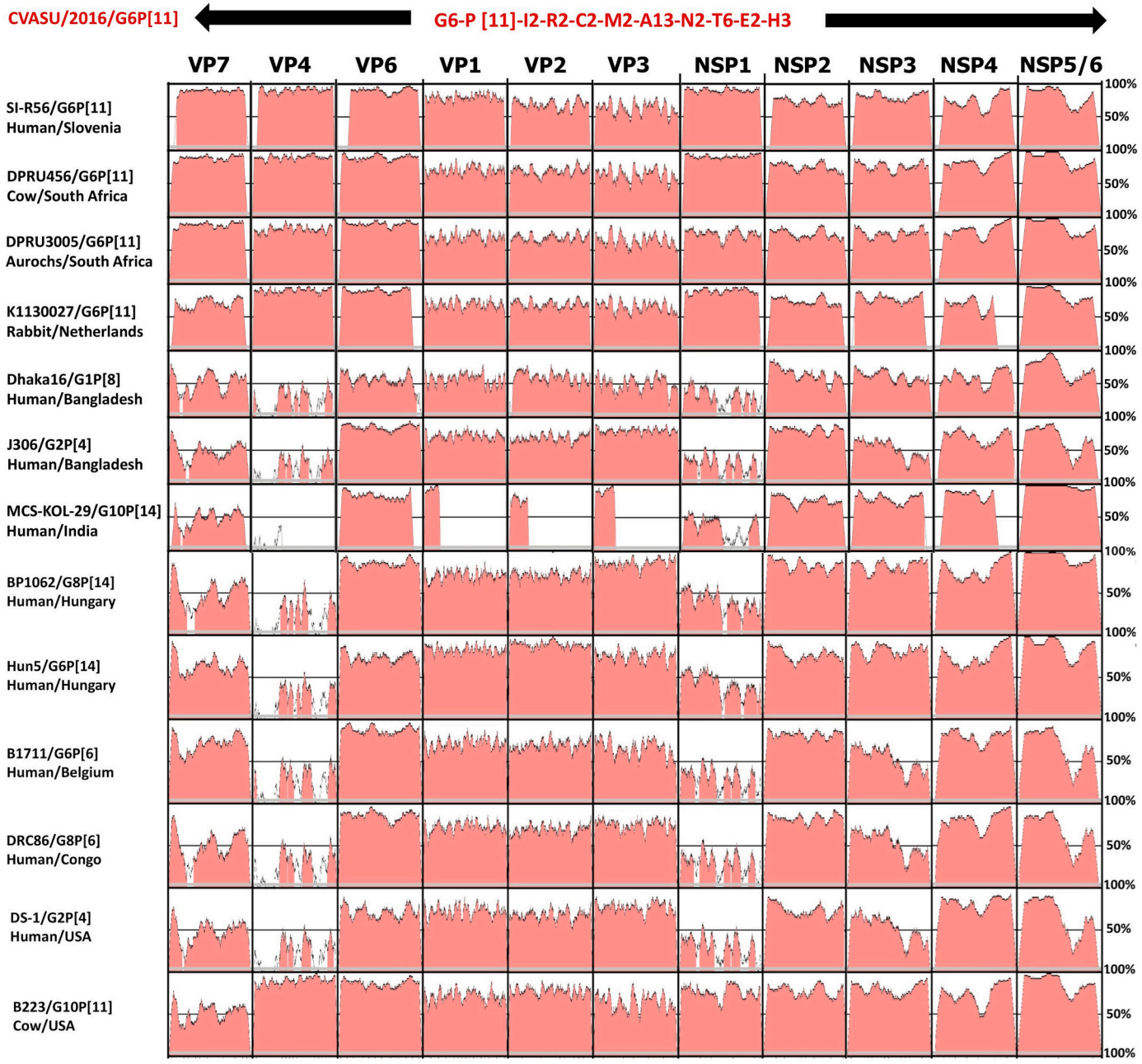

Fig. 1. mVISTA whole genome nucleotide alignment comparing the BD_ROTA_CVASU isolate with 13 other rotavirus strains (DPRU456, DPRU3005 and B223) of bovine, (SI-R56, Dhaka16, J306, MCS-Kol-29, BP1062, Hun5, BP1711, DRC86 and DS-1) of human and K1130027 of rabbit strains from 10 different genotypes which constitute a genotype composition of each of the 13 strains Percentage (right y-axis) indicates sequence identity. The red filled histogram demonstrates nucleotide similarity percentages of BD_ROTA_CVASU genome with mentioned strains.

typical DS-1-like genetic backbone possessed by DS-1 like strains reported elsewhere in the world. The BLAST searches have shown that 6 out of 11 genome segments including gene encoding VP7 capsid protein had highest nucleotide identity (95%–99%) with RVA strains isolated from cattle while at least 4 segments had maximum sequence similarity with human isolates. Interestingly, the VP4 gene has shown closest match with a RVA strain of G6P[11] genotype isolated from Rabbits (Fig. 1). Although for most genome segments the closest match was identified from strains circulating in India, however some segments had closer relationship with strains isolated in Europe and Africa. RVA strains of different G and P genotypes including G6, G10, P[5], P[10] and P[11] have shown nucleotide match however, all of them had DS-1 like genome constellation even though whole genome constellation were not available for several BLAST match strains (Fig. 1).

3.4. Comparative analyses of whole genome constellation as visualized by mVISTA module

Using mVISTA module we aligned whole genome constellation of BRA/CalfWt/Bangladesh/CVASU/2016/G6P[11] with 13 RVA strains of various genotypes having DS-1 genomic backbone and circulating in bovine and human hosts from different parts of the world (Fig. 1). Unsurprisingly, the highest overall nucleotide sequence conservation (75–95% nt) was detected in genomes with G6P[11] genotype irrespective to the host or location. Despite having same DS-1 like genomic backbone the genome segments encoding VP7, VP4 and NSP1 proteins demonstrated significant nucleotide variation (<50 % nt identity) in previously reported strains circulating in human hosts in Bangladesh and India as it was reflected by their corresponding genotype association (Fig. 1; Supplementary Table 2). However, the relative constellation conservation of the CVASU strain with human and animal associated strains from different geographical location highlights that this particular RVA strain may have been circulating in these hosts for a long time.

3.5. Phylogenetic analyses

We constructed phylogenetic trees using the full-length gene sequence for each of the 11 gene segments because phylogenetic analysis of RVA nucleotide sequences makes it possible to obtain direct evidence of their relatedness to not only those of other RVA strains but also within the same genotype. The complete sequence of gene segment encoding VP1 to VP4 and VP6 of BD_ROTA_CVASU isolate was compared with corresponding gene segments publicly available in GenBank (Supplementary Figs. 2–6). In phylogenetic analysis, VP7 gene of BD_ROTA_CVASU strain clustered with various human, bovine and swine strains (Fig. 2). BD_ROTA_CVASU strain closely clustered with the Japanese, USA and Italian human strains as well as Canadian bovine strain. Our G6 strain also showed relation with the caprine G6 strain of Bangladesh and different Indian G6 bovine strain but formed separate cluster from BD_Rota_CVASU isolate.

Unlike the structural viral proteins, the non-structural proteins (NSPs) were generally more closely related to known RVA strains. The NSP4 gene of BD_ROTA_CVASU isolate was shown to be closely related to the several Indian bovine strains and Thai bovine strain (Fig. 3). Our isolate BD_ROTA_CVASU found belonging to E2 genotype and distantly related to the other genotype like E1, E9 and E14 were shown in the phylogenetic tree. It was exhibited that Bangladeshi human strain clustered in the different lineage of E2 genotype. Phylogenetic tree constructed from the nucleotide sequences of the NSP1 to NSP3 and NSP5 gene of isolate BD_ROTA_CVASU and representative RVA strains has been shown in Supplementary Figs. 7–10 respectively.

3.6. Recombination analysis

Global recombination analysis using all publicly available genome demonstrated significant recombination events in NSP4 segments where rest of the gene segments had no significant breakpoints with BD_ROTA_CVASU strain. Regarding NSP4 gene segment, five recombinant events were detected statistically significant which demonstrate intermixing of human and bovine RVA strains from different geographical origin (Table 1, Supplementary Table 2). BD_ROTA_CVASU strain was detected as recombinant sequence where bovine and human strains from South Africa and Thailand respectively were acting as major parents (JN831218 and MH060117). At the same time, contribution of BD_ROTA_CVASU strain as major parent and minor parent also highlighted intermingling of human RVA strains with bovine RVA to form recombinant sequences (AC: EF672568, AB361290 and AB326293). All the sequences significant in recombinant breakpoints belongs to E1 and E2 sub-genotype (Fig. 3).

4. Discussion

The whole genome constellation of RVA provides valuable insights into the genetic diversity, spatiotemporal distribution and evolution of circulating strains in particular host niche while also provide opportunity to explore plausible spillover events and transmission dynamics that shape its global epidemiology. However, determining the complete sequence of the 18.55-kb segmented genome using traditional primer-based amplification and Sanger sequencing strategies can be a daunting task as the initial PCR may not predict chimeric genotype from genetic reassortment and recombination event or may result in sequencing failure in the case of atypical or un-typeable RVA strains (Atchison et al., 2010; Pitzer et al., 2011). With the advent of Next-generation sequencing (NGS) technology these issues have been largely solved and the numbers of complete genome constellations with no prior genotype information has increased drastically over the past decade. Here, we characterised the complete genomic constellation of a bovine strain of *Rotatvirus A* with a highly prevalent genotype G6P [11] from commercial cattle farm in Bangladesh using NGS based whole genome shotgun sequencing and downstream comparative and phylogenomic analysis.

Among the common bovine rotavirus strains, G6P[11] has long been the most prevalent G-P genotype combination in both developed and developing countries worldwide (Rahman et al., 2010). Most gene segments of bovine rotavirus strains cluster within the same corresponding genotypes, regardless of geographic origin or time of isolation, therefore considered as typical gene segments of bovine rotaviruses (Komoto et al., 2016; Matthijnssens et al., 2009a). Similarly, other members of the family Bovidae or the order Artiodactyla have recently been shown to share a largely conserved consensus genotype constellation with bovine rotaviruses (Matthijnssens et al., 2009b). Phylogenetic analysis of all the eleven segments of BD_ROTA_CVASU strain clustered with respective gene segments with other south and south-east Asian strains, however some African, American, and the European strains were also clustered the same lineage demonstrating high degree of genetic intermixing among different strains. Notably there was close phylogenetic relationship of the individual gene segments of BD_ROTA_CVASU strain to the Indian isolates. This is not unexpected as India shares the fifth-longest international land border of about 4,096 km with Bangladesh and there are frequent reports of cross-border disease transmission through legal and illegal livestock trade (Gongal et al., 2022).

Analysis of complete genome sequence have demonstrated evidence of naturally occurring frequent reassortment between RVA strains of humans and animals' origin (Ghosh et al., 2010; Matthijnssens et al., 2009a; Rahman et al., 2010; Steyer et al., 2013). The close proximity or sharing of similar dwellings between animal and human interfaces promote interspecies transmission of rotaviruses, particularly in low socioeconomic situations (Bwogi et al., 2017). Such transmission risk may exist in water reservoirs or foods contaminated by the faeces of infected animals. Genetic intermixing of distinct host adapted genotypes can result in a progeny virus with a genetic composition compatible for

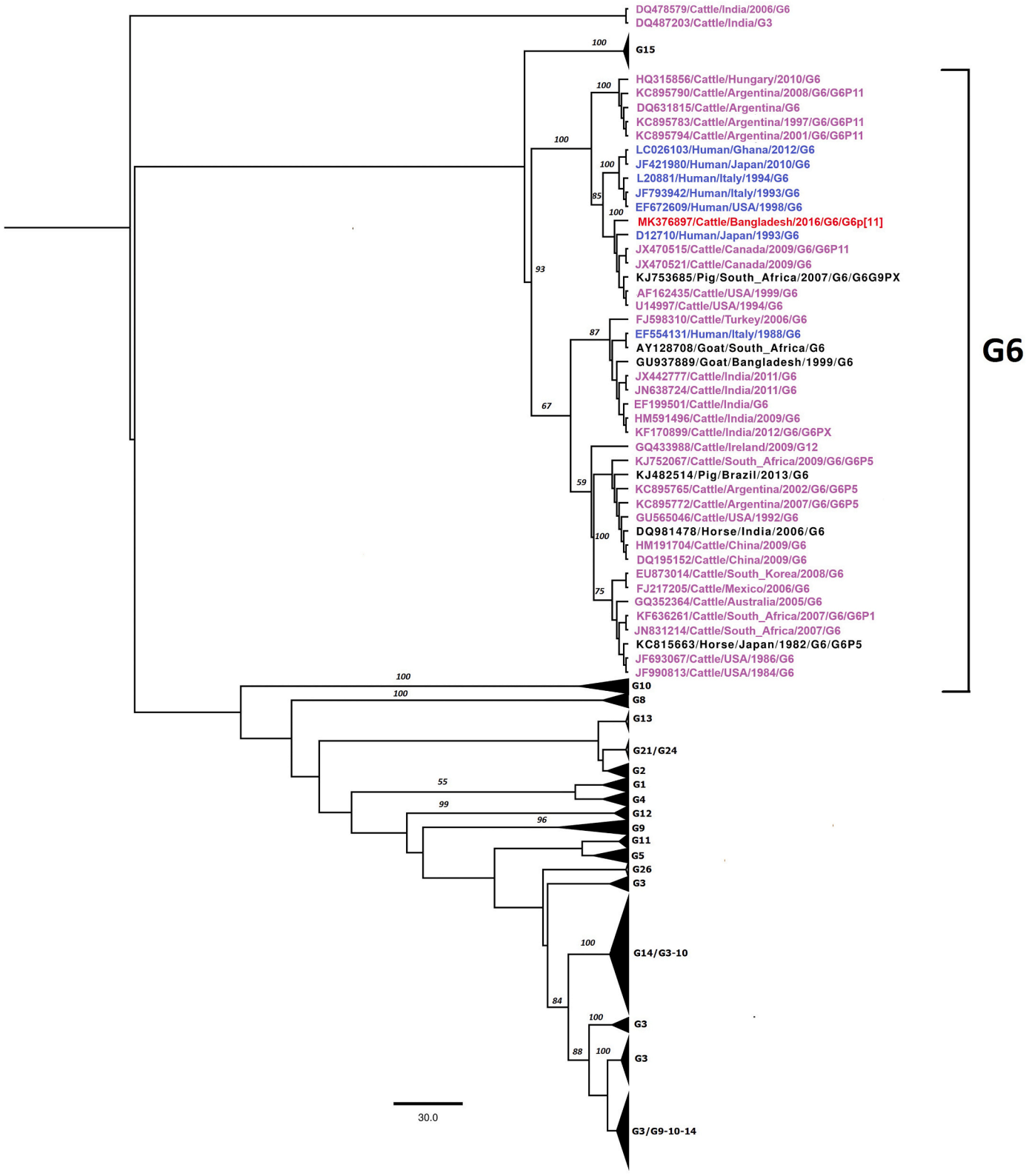

Fig. 2. Phylogenetic tree constructed from the nucleotide sequences of the VP7 gene of isolate BD_ROTA_CVASU and representative RVA strains. In the tree, the position of isolate BD_ROTA_CVASU is red colour human strains are shown in blue, bovine strains are in violet while other strains are shown in black colour. The trees were constructed using the maximum likelihood method included in the FigTree software package with 100 replicate trials and rooted with the sequences of different genotypes. Bootstrap values of <50% are not shown. Scale bars: 30.

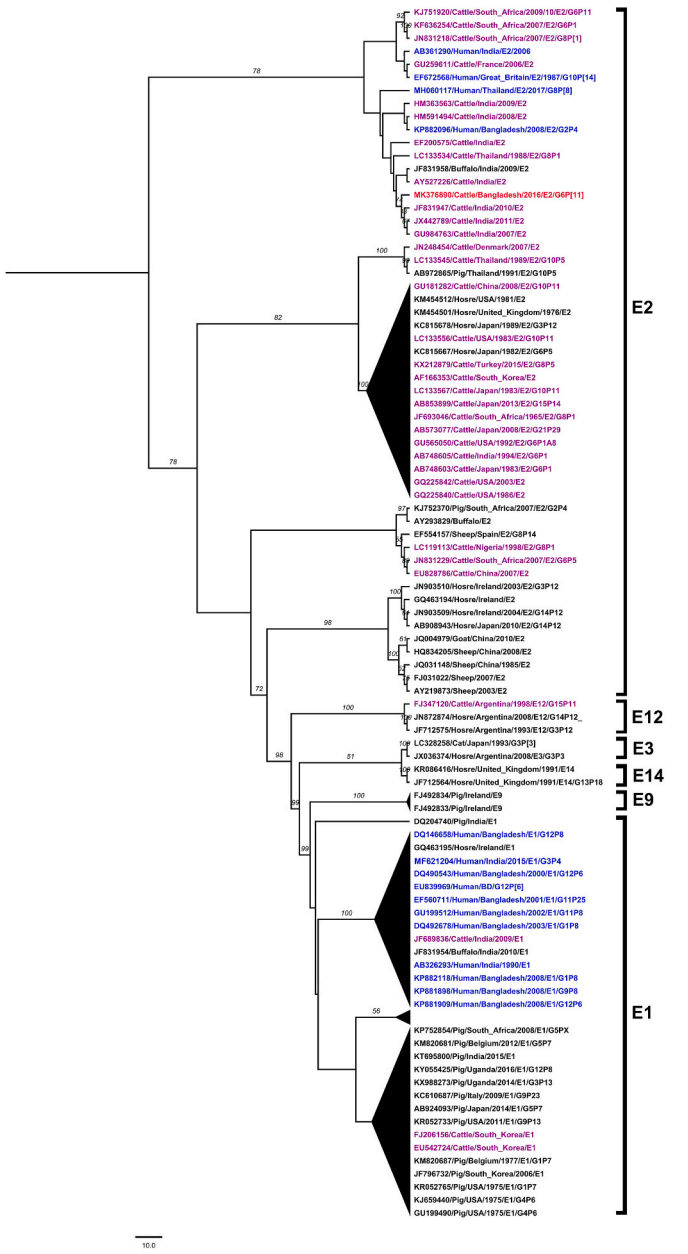

Fig. 3. Phylogenetic tree constructed from the nucleotide sequences of the NSP4 gene of isolate BD_ROTA_CVASU and representative RVA strains. In the tree, the position of isolate BD_ROTA_CVASU is red colour, human strains are shown in blue, bovine strains are in violet while other strains are shown in black colour. The trees were constructed using the maximum likelihood method included in the FigTree software package with 100 replicate trials and rooted with the sequences of different genotypes. Bootstrap values of <50% are not shown. Scale bars: 10.

replication and spread in the human population (Matthijnssens et al., 2010a). Sporadic transmission of un-reassorted or reassorted BRV strains to human population was reported previously (Doan et al., 2013). One study documented detection of bovine origin G6P [1] strain from a child in Israel with gastroenteritis (Doan et al., 2013). Similarly, rare bovine like human VP4 mono-reassortment G6P[8] RVA was detected in a 7-month-old human child with acute diarrhea in Bangladesh (Afrad et al., 2013). In such instances, complete genome constellation data of locally circulating RVA strains from different host niche and interfaces provide critical understanding on zoonotic transmission, allowing informed intervention to mitigate public health risks. Unfortunately, despite being highly prevalent in Bangladesh and other low-mid income countries in south Asia, the constellation data from RVA strains circulating in commercial livestock sector in scarce and mostly reliant upon partial VP7/VP4 genotyping for surveillance. Likewise, the highly prevalent G6P [11] genotype was previously reported in cattle from different geographical locations of Bangladesh (Hossain et al., 2020; Uddin Ahmed et al., 2022) based on partial sequences without being characterised in detail. This study sequenced the complete genome constellation of a bovine rotavirus strain circulating in Bangladesh for the first time with a highly prevalent G6P [11] genotype and DS-1 like genomic backbone allowing detailed genealogical characterisation. However, more NGS of BRV from different geographical areas of Bangladesh might help to demonstrate a well scenario of circulating BRV strains.

Some features in rotavirus biology such as co-infections with multiple stains from similar hosts make recombination events not only possible, but also relatively plausible (Hoxie and Dennehy, 2020). Recombination events are also commonly observed with other RNA viruses such as avian influenza, Newcastle disease virus and poliovirus (Acevedo et al., 2018; He et al., 2009; Satharasinghe et al., 2016; Suarez et al., 2004), however the frequency of recombination in RNA viruses varies from species to species. It was previously thought that intra-segmental recombination was not considered a significant driver of rotavirus genetic diversity and evolution (Woods, 2015). However, later studies showed evidence of intragenic homologous recombination with long-term effect on rotavirus diversity and evolution (Hoxie and Dennehy, 2020; Phan et al., 2007). We employed rigorous recombination analysis involving all of the eleven gene segments of BD_ROTA_CVASU strain all full-length sequence data available in public database up until September 2022. Surprisingly the analyses revealed no significant recombination events for 10 segments except NSP4 gene (Table 1). The NSP4 gene segment of BD_ROTA_CVASU strain (GenBank AC No: MK376890) had two large recombination breakpoints ~690–700 bp in size, where genetic intermixing of human and bovine strains was highly evident. While being strongly supported by multiple statistical models these recombination breakpoints could not fully resolve the evolutionary source of genetic intermixing as the minor parents were unknown in both cases. However, there was strong evidence of human strains acting either as major (MH060117) or minor parents (DQ490543) in these events. Similar scenario presented breakpoint 3–4 where NSP4 gene segment from BD_ROTA_CVASU strain was involved either as major or minor parent into evolution of three different human strains from India and Great Britain. Although these events demonstrated potential genomic intermixing between human and animal strains of RVAs these events lack spatial-temporal signal for recent divergence through coinfection. False positives by recombination detection software are common (Bertrand et al., 2016) among closely related strains. Given that, recombination events are likely occur between closely related strains in close spatial/temporal proximity and genetic compatibility, so caution should be used in inferring events. However, we employed robust strategies to tackle the shortcoming and picked up events only supported by at least 3 different models (Hoxie and Dennehy, 2020). In this context, detecting frequent and diverse recombination signals only in NSP4 gene segment while other structural protein coding genes VP7/VP4 showed no signal rather interesting. The RVA non-structural protein NSP4 is the viral enterotoxin that acts as viroporin (Pham et al., 2017) through integrin receptors on uninfected enterocytes, which in turn causes and increases intracellular Ca^{2+}-concentrations clinically resulting in hypersecretory diarrhea (Hyser et al., 2010). Recombination between similar segments of human and animal RVA strains could trigger the pathogenicity of such recombinant strains, followed by long-time evolutionary effects. This hypothesis requires further clarification through cell biology experiments which is beyond the scope of this study. The recombination events therefore highlight the deep evolutionary relatedness among human and bovine rotavirus strains. These findings could support the hypothesis that human DS-1-like and bovine (artiodactyl) strains may have shared a

Table 1
Evidence of recombination events throughout Rotavirus A NSP4 segment alignment.

Event number	Recombinant sequence(s)	Potential major parent(s)	Potential minor parent(s)	Breakpoint positions	Supporting methods[b]	*p*-value
1	MK376890/Cattle/BD/2016/G6P[11]	JN831218/Bovine/ZA/2007/G8P[1]	DQ490543/Human/BD/2000/G12P[6][a]	37–734	**R**, G, S, 3S	2.35×10^{-10}
2	MK376890/Cattle/BD/2016/G6P[11]	MH060117/Human/TH/2017/G8P[8]	LC328258/Cat/JP/1993/G3P[3][a]	80–734	R, G, **S**, 3S	8.20×10^{-08}
3	EF672568/Human/GB/1987/G10P[14]	MK376890/Cattle/BD/2016/G6P[11]	MF621204/Human/IN/2015/G3P[4]	526–1019	R, G, S, **3S**, M, C	3.68×10^{-24}
4	AB361290/Human/IN/2006	MK376890/Cattle/BD/2016/G6P[11]	EU839969/Human/BD/G12P[6]	590–725	R, G, S, **3S**, M, C	3.19×10^{-12}
5	AB326293/Human/IN/1990	MF621204/Human/IN/2015/G3P[8]	MK376890/Cattle/BD/2016/G6P[11][a]	631–704	**R**, G, S	1.10×10^{-04}

[a] The sequence was used to infer the existence of a missing parental sequence. Only one parent and a recombinant need to be in the alignment for a recombination event to be detectable. Here, potential minor and major parents are contributing the smaller and larger fractions respectively of the recombinant sequence.
[b] For supporting methods, R, G, M, C, S and 3S stand for RDP, GENECONV, Maxchi, Chimaera, SiSscan, 3Seq respectively with the strongest *p*-value shown for the detection method in bold.

common ancestor, and eventually evolved over time (Matthijnssens et al., 2008).

5. Conclusion

The genome constellation of BD_ROTA_CVASU (G6-P[11]-I2-R2-C2-M2-A13-N2-T6-E2-H3) represents a bovine strain possessing DS-1 like genomic backbone with deep evolutionary relatedness with human strains demonstrated by frequent recombination events in NSP4 gene. To best of our knowledge this is the first complete genome of a dominant bovine rotavirus strain circulating in commercial cattle industry in Bangladesh.

CRediT authorship contribution statement

Shama Ranjan Barua: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Tridip Das:** Writing – review & editing, Writing – original draft, Formal analysis. **Tofazzal Md Rakib:** Writing – review & editing, Investigation. **Babu Kanti Nath:** Writing – review & editing. **Suman Das Gupta:** Writing – review & editing. **Subir Sarker:** Writing – review & editing, Investigation. **Sharmin Chowdhury:** Writing – review & editing, Conceptualization, Supervision, Funding acquisition. **Shane R. Raidal:** Writing – review & editing. **Shubhagata Das:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data used are available in the manuscript and supplementary file.

Acknowledgments

The authors are grateful to Clinical Pathology Lab, Chattogram Veterinary and Animal Sciences University, Bangladesh and Dr Subir Sarker, La Trobe University, Australia for providing laboratory facilities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2024.110195.

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