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Research paper

# Genetic analysis of human rotavirus C: The appearance of Indian–Bangladeshi strain in Far East Asian countries

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ABSTRACT

Rotaviruses C (RVCs) circulate worldwide as an enteric pathogen in both humans and animals. Most studies of their genetic diversity focus on the VP7 and VP4 genes, but the complete genomes of 18 human RVCs have been described in independent studies. The genetic background of the Far East Asian RVCs is different than other human RVCs that were found in India and Bangladesh. Recently, a RVC detected in 2010 in South Korea had genetic background similar to the Indian–Bangladeshi RVCs. This study was undertaken to determine the whole genome of eight Japanese RVCs detected in 2005–2012, and to compare them with other human and animal global RVCs to better understand the genetic background of contemporary Far East Asian RVC. By phylogenetic analysis, the human RVCs appeared to be distinct from animal RVCs. Among human RVCs, three lineage constellations had prolonged circulation. The genetic background of the Far East Asian RVC was distinguished from Indian–Bangladeshi RVC as reported earlier. However, we found one Japanese RVC in 2012 that carried the genetic background of Indian–Bangladeshi RVC, whereas the remaining seven Japanese RVCs carried the typical genetic background of Far East Asian RVC. This is the first report of the Indian–Bangladeshi RVC in Japan. With that observation and the reassortment event of human RVCs in Hungary, our study indicates that the RVCs are spreading from one region to another.



## 1. Introduction

Rotavirus, a member of the family *Reoviridae*, is a leading cause of acute gastroenteritis in young children worldwide. Understanding the epidemiology of these viruses is critical to public health efforts to control outbreaks. The viral genome consists of 11 segments of double-stranded RNA that encode six structural viral proteins (VPs) and six nonstructural proteins (NSPs) (Estes and Greenberg, 2013). Based on the antigenic properties of VP6, rotaviruses have been subdivided into eight serological groups (A–H) (Matthijnssens et al., 2012; Ramig et al., 2005). Groups A, B, C and H rotaviruses are found in humans and animals, and rotaviruses of groups D, E, F and G have been found only in animals to date (Estes and Greenberg, 2013; Matthijnssens et al., 2012).

Rotaviruses C (RVCs) were first isolated in piglets in 1980 (Saif et al., 1980) and in humans in 1982 (Rodger et al., 1982). Since then, they have been detected in humans and animals from many countries. RVCs circulate throughout the world but are much less common than

*Abbreviations:* VP, viral protein; NSP, non-structural protein; G, the suffix for the VP7 genotype deriving from Glycoprotein; P, the suffix for the VP4 genotype deriving from Protease sensitive protein; I, the suffix for the VP6 genotype deriving from Intermediate capsid shell; R, the suffix for the VP1 genotype deriving from RNA-dependent RNA polymerase; C, the suffix for the VP2 genotype deriving from Core shell protein; M, the suffix for the VP3 genotype deriving from Methyltransferase; A, the suffix for the NSP1 genotype deriving from Interferon Antagonist; N, the suffix for the NSP2 genotype deriving from NTPase; T, the suffix for the NSP3 genotype deriving from Translation enhancer; E, the suffix for the NSP4 genotype deriving from Enterotoxin; H, the suffix for the NSP5 genotype deriving from pHosphoprotein; dNTPs, deoxynucleotide triphosphates; tMRCA, the time of the most recent common ancestor; GTR, General Time Reversible; HKY, Hasegawa–Kishino–Yano; TN93, Tamura–Nei; T92, Tamura 3-parameter.

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rotaviruses A (RVAs). Like RVAs, the RVCs have two outer capsid proteins, VP7 and VP4, that possess neutralization-specific epitopes. Glycoprotein VP7 and protease-sensitive VP4 define the G and P types, respectively. While 10 G genotypes and 8 P genotypes have been reported so far, only G4 and P[2] have been found in human RVCs (Jeong et al., 2015; Martella et al., 2007; Marthaler et al., 2013; Marton et al., 2015a; Marton et al., 2015b; Suzuki et al., 2015).

Many studies have examined the evolution of RVAs, but the evolutionary patterns for the RVCs are not well understood. Therefore, it remains to be determined as to whether the RVCs exhibit similar evolutionary patterns as RVAs. Recently, a genotyping system was developed for the 11 genome segments of RVAs, based on nucleotide sequence identity cutoff values (Matthijnssens et al., 2011; Matthijnssens et al., 2008). It provides an excellent system for detection reassortment events and examining the evolution of rotaviruses at a whole-genome level. That approach was also applied to 11 genome segments of RVCs (Soma et al., 2013; Yamamoto et al., 2011). According to RVC classification, the genotypes of the human and animal RVCs were different (Soma et al., 2013). Except for the VP3 gene, which was assigned into two genotypes M2 and M3, the remaining 10 genes of human RVCs were assigned to a single genotype (i.e., G4, P[2], I2, R2, C2, A2, N2, T2, E2 and H2 for VP7, VP4, VP6, VP1, VP2, NSP1, NSP2, NSP3, NSP4 and NSP5, respectively) (Yamamoto et al., 2011).

The genetic diversity in the RVCs has been examined in several studies, and most were based on the VP7 and VP4 genes. So far the complete genomes have been analyzed only for one porcine RVC, one canine RVC, seven bovine RVCs and eighteen human RVCs (Baek et al., 2013; Chen et al., 2015; Chen et al., 2002; Marton et al., 2015a; Marton et al., 2015b; Mawatari et al., 2014; Soma et al., 2013; Yamamoto et al., 2011; Zhirakovskaia et al., 2016). These studies showed that the genetic background of RVCs was different according to host species. Among human RVCs, the genetic backgrounds of the Far East Asian strains were reported to be different from other human RVCs which were detected in India and Bangladesh and including the putative unique genotype of the VP3 gene as well as minor sequence variations in other genes (Yamamoto et al., 2011). However, recently Baek et al. (2013) described a Far East Asian RVC detected in 2010 in South Korea that has a genetic background similar to the Indian and Bangladeshi strains. Moreover, in Hungary, Marton et al. (2015a) reported the occurrence of reassortant human RVCs carrying the Indian and Bangladeshi NSP4 gene on the Far East Asian strain genetic background.

In the present study, we sought to determine the whole genome of eight Japanese RVCs: five strains detected in 2005–2008 and three strains detected in 2011–2012. We then conducted phylogenetic analysis on the 11 genome segments of these strains along with those of the all human and animal global RVCs deposited in GenBank during last 27 years (1988–2014) to better understand the genetic background of contemporary Far East Asian RVCs.

## 2. Materials and methods

### 2.1. Specimen and sequence collection

Complete genome sequences were determined for eight human Japanese RVCs, including HI-49 (Hiroshima prefecture, 2006), HO-61, HO-62 and HO-63 (Hokkaido prefecture, 2005), HO-64 (Hokkaido prefecture, 2008), HO-65 (Hokkaido prefecture, 2011), YA-27 (Yamaguchi prefecture, 2011), and OS-270 (Osaka prefecture, 2012). The HI-49 specimen was collected from a sporadic case of diarrhea, and the other seven specimens were collected from outbreaks of diarrhea.

We searched the GenBank database for all available RVC sequences by using the BLAST program (http://blast.ncbi.nlm.nih.gov/) with the sequences of our RVCs as the query sequences for all 11 genome segments. We increased the "Max target sequences" option in the BLAST program to 1000 to ensure inclusion of all relevant sequences from the hits, and retrieved 298-, 120-, 202-, 27-, 29-, 27-, 28-, 29-, 29-, 81-, and 34-nucleotide sequences for VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4 and NSP5 genes of human and animal RVCs, respectively. Among these sequences, we obtained 11 genome segments that were possessed by eighteen human RVCs, one porcine RVC, one canine RVC and seven bovine RVCs that had been collected in the last 27 years (1988–2014) from around the world. In addition, we also collected other 55-, 27-, 20-, and 42-nucleotide sequences for VP7, VP4, VP6, and NSP4 genes of human RVCs that contained the open reading frame of each segment, respectively.

### 2.2. RNA extraction

Viral RNA was extracted using TRIzol LS Reagent (Life Technologies, Grand Island, NY, USA) and Direct-zol RNA MiniPrep Kit (ZYMO Research, USA), according to the manufacturer's instructions. Briefly, 240 μl of TRIrizol LS Reagent was added to 80 μl of stool suspension and homogenized by vortexing. After a 5-min incubation at room temperature, 320 μl of ethanol was added, and the mixture was directly loaded into the spin column provided in the kit. The spin column was centrifuged at 12,000 $\times g$ for 1 min and then washed according to the manufacturer's instructions. Finally, the purified RNAs were eluted with 40 μl of DNase/RNase-free water.

### 2.3. cDNA library building and Illumina MiSeq sequencing

cDNA library sequencing and Illumina MiSeq sequencing were performed as described (Dennis et al., 2014). Briefly, a 200-bp fragment library ligated with bar-coded adapters was constructed for individual strains with a NEBNext Ultra RNA library Prep Kit for Illumina v1.2 (New England Biolabs, MA, USA), according to the manufacturer's instructions. The library was purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, USA). The quality of the purified cDNA libraries was assessed on a MultiNA MCE-202 bioanalyzer (Shimadzu, Japan). Nucleotide sequencing was performed on an Illumina MiSeq sequencer (Illumina) with a MiSeq Reagent Kit v2 (Illumina, CA, USA) to generate 151 paired-end reads. Data analysis was carried out using CLC Genomics Workbench v7.0.3 (CLC Bio). Contigs were assembled from the obtained sequence reads by de novo assembly. The contigs included the rotavirus sequences and other sequences, such as human and bacterial sequences. The full-length nucleotide sequence of each gene segment of eight Japanese RVCs was obtained by using Basic Local Alignment Search Tool (BLAST) against local data in CLC Genomics Workbench with the assembled contigs as query sequences and 11 genome segments of reference RVC as target sequence.

### 2.4. Genotyping

The genotype of each of the 11 genome segments was determined according to predefined nucleotide sequence identity cutoff values, which were adapted as described (Jeong et al., 2015; Marthaler et al., 2013; Soma et al., 2013; Suzuki et al., 2014; Yamamoto et al., 2011).

### 2.5. Phylogenetic analysis

The near-full-length genome sequences determined in this study were aligned with the sequences that were obtained from the GenBank database by the MAFFT multiple sequence alignment software program, version 7.0 (Katoh et al., 2002). A maximum likelihood tree was constructed for each genome segment, based on the nucleotide sequences of the eight Japanese RVCs and those of the corresponding genome segments of the human and animal RVCs that had the sequences available in GenBank database. The best substitution models were selected based on the corrected Akaike information criterion value as implemented in MEGA6 (Tamura et al., 2013). Models used in this study were TN93 + G (VP7), TN93 + I (NSP3), TN93 + G + I (VP2), T92 + G

**Table 1**
Ranges of nucleotide identity of 8 newly identified Japanese RVCs with reference human and animal RVCs.

| Genes | Nucleotide identity between OS-270 and Indian–Bangladeshi RVCs (%) | Nucleotide identity between 8 newly identified Japanese RVCs and reference human and animal RVCs (%) | | | | Cut-off values (%) |
|---|---|---|---|---|---|---|
| | | Human RVC | Porcine | Bovine | Canine | |
| VP7 | 97.55–100 | 93.55–100 | 80.04–80.99 | 65.88–68.85 | 70.73–71.46 | 85(*) and 84 (**) |
| VP4 | 99.36–97.43 | 95.18–99.95 | 65.51–66.02 | 61.67–63.43 | 63.73–64.28 | 83(***) and 77(**) |
| VP6 | 99.10–97.35 | 95.54–100 | 77.53–81.26 | 77.52–78.90 | 76.38–77.64 | 90(***), 87(****) and 85(**) |
| VP1 | 99.13–96.74 | 93.62–99.88 | 82.99–83.11 | 75.75–77.14 | 76.26–76.53 | 86(**) |
| VP2 | 98.43–96.13 | 94.14–99.70 | 80.10–80.91 | 73.68–75.82 | 73.69–74.71 | 84(**) |
| VP3 | 90.94–98.34 | 80.80–99.76 | 78.58–83.24 | 71.98–74.66 | 71.28–74.65 | 86(**) |
| NSP1 | 94.86–98.72 | 91.99–99.92 | 53.93–55.06 | 53.71–56.41 | 56.71–56.90 | 74(**) |
| NSP2 | 96.71–97.39 | 93.13–100 | 85.33–86.80 | 77.34–79.29 | 76.55–77.02 | 89(**) |
| NSP3 | 97.71–96.40 | 91.80–100 | 70.97–72.70 | 71.53–74.19 | 73.67–75.55 | 80(**) |
| NSP4 | 96.28–98.70 | 93.49–100 (#) | 49.63–53.08 | 50.75–57.99 | 54.15–57.30 | 71(**) |
| NSP5 | 97.90–96.54 | 91.27–99.84 | 71.20–71.96 | 67.74–70.56 | 71.07–72.54 | 79(**) |

Cut-off values assigning various genotypes were adapted from previous publications: (*) Marthaler et al. (2013), (**) Soma et al. (2013), (***) Jeong et al. (2015) and (****) Suzuki et al. (2014), (#) range of nucleotide identity between 8 newly identified Japanese RVCs and reference human E2 genotype.

**Table 2**
The genotype constellation of eight newly identified Japanese RVCs as well as those of 27 human and animal RVCs for which all 11 genome segments were available in the GenBank.

| | Name of RVC | Country of isolation | Year of isolation | VP7 | VP4 | VP6 | VP1 | VP2 | VP3 | NSP1 | NSP2 | NSP3 | NSP4 | NSP5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human | Wu82 | China | 2001 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | OH567 | Japan | 2003 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | HO–61 | Japan | 2005 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | HO–62 | Japan | 2005 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | HO–63 | Japan | 2005 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | HI–49 | Japan | 2006 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | YNR001 | Japan | 2007 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | BK0830 | Japan | 2008 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | HO–64 | Japan | 2008 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | HO–65 | Japan | 2011 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | YA–27 | Japan | 2011 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | SZ272 | China | 2011 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | SZ94 | China | 2011 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | ERN6210 | Hungary | 2013 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | ERN6216 | Hungary | 2013 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | ERN6233 | Hungary | 2013 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | Chungnam | South Korea | 2014 | G4 | P[2] | I2 | R2 | C2 | M3 | A2 | N2 | T2 | E2 | H2 |
| Human | Bistol | United Kingdom | 1988 | G4 | P[2] | I2 | R2 | C2 | M2 | A2 | N2 | T2 | E2 | H2 |
| Human | v508 | India | 2005 | G4 | P[2] | I2 | R2 | C2 | M2 | A2 | N2 | T2 | E2 | H2 |
| Human | BS347 | Bangladesh | 2005 | G4 | P[2] | I2 | R2 | C2 | M2 | A2 | N2 | T2 | E2 | H2 |
| Human | Omsk08–386 | Russia | 2008 | G4 | P[2] | I2 | R2 | C2 | M2 | A2 | N2 | T2 | E2 | H2 |
| Human | Nsk08–3414 | Russia | 2008 | G4 | P[2] | I2 | R2 | C2 | M2 | A2 | N2 | T2 | E2 | H2 |
| Human | Omsk08–436 | Russia | 2008 | G4 | P[2] | I2 | R2 | C2 | M2 | A2 | N2 | T2 | E2 | H2 |
| Human | Nsk09–B43 | Russia | 2009 | G4 | P[2] | I2 | R2 | C2 | M2 | A2 | N2 | T2 | E2 | H2 |
| Human | CAU 10–312 | South Korea | 2010 | G4 | P[2] | I2 | R2 | C2 | M2 | A2 | N2 | T2 | E2 | H2 |
| Human | OS–270 | Japan | 2012 | G4 | P[2] | I2 | R2 | C2 | M2 | A2 | N2 | T2 | E2 | H2 |
| Porcine | Cowden | United Kingdom | 1980 | G1 | P[1] | I1 | R1 | C1 | M1 | A1 | N1 | T1 | E1 | H1 |
| Bovine | Shintoku | Japan | 1991 | G2 | P[3] | I3 | R3 | C3 | M4 | A3 | N3 | T3 | E3 | H3 |
| Bovine | Y/03 | Japan | 2003 | G2 | P[3] | I3 | R3 | C3 | M4 | A3 | N3 | T3 | E3 | H3 |
| Bovine | Y/1/04 | Japan | 2004 | G2 | P[3] | I3 | R3 | C3 | M4 | A3 | N3 | T3 | E3 | H3 |
| Bovine | Y/3/04 | Japan | 2004 | G2 | P[3] | I3 | R3 | C3 | M4 | A3 | N3 | T3 | E3 | H3 |
| Bovine | Y/08 | Japan | 2008 | G2 | P[3] | I3 | R3 | C3 | M4 | A3 | N3 | T3 | E3 | H3 |
| Bovine | Y/10 | Japan | 2010 | G2 | P[3] | I3 | R3 | C3 | M4 | A3 | N3 | T3 | E3 | H3 |
| Bovine | Toyama | Japan | 2012 | G2 | P[3] | I3 | R3 | C3 | M4 | A3 | N3 | T3 | E3 | H3 |
| Canine | 174 | Hungary | 2012 | G10 | P[8] | I8 | R(–) | C(–) | M(–) | A(–) | N(–) | T(–) | E(–) | H(–) |

RVCs determined in this study: name in red.
(-) the sequences are available in GenBank but genotype number has not been decided by Marton et al. (2015).

(VP6, NSP2 and NSP4), GTR + G + I (NSP1, VP3), HKY + G (NSP5), and HKY + G + I (VP1, VP4).

### 2.6. Bayesian evolutionary analysis using BEAST

The evolutionary rates and the time of most recent common ancestors were determined for all 11 genome segments of the human RVCs with the Bayesian Markov chain Monte Carlo (MCMC) method implemented in BEAST v1.8.1 (Drummond et al., 2012). Models used for BEAST analyses of human RVCs were T92 + G (VP2, VP4, VP6, VP7, NSP1 and NSP2), T92 + I (NSP4), and HKY + G (VP1, VP3, NSP3 and NSP5). Strict clock and coalescent exponential growth models (Drummond et al., 2002) were used. MCMC runs were carried out for 200 million generations to achieve convergence with sampling every 1000 steps. Convergence was assessed from effective sample size after a 10% burn-in using Tracer software v1.6 (http://tree.bio.ed.ac.uk/software/tracer/). Only parameters with an effective sample size of greater than 200 were accepted. Maximum clade credibility trees were annotated with the Treeannotator and viewed with FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

### 2.7. Nucleotide sequence accession numbers

The nucleotide sequences described in this study were deposited in the DDJB/GenBank/EMBL databases under the accession numbers LC129042 to LC129129.

## 3. Results

We determined nearly complete nucleotide sequences of the 11 genome segments of all eight Japanese strains. The lengths and nucleotide regions of sequences obtained are shown in Supplement 1. Based on the nucleotide identities between newly identified Japanese RVCs with reference human and animal RVCs (Table 1), all eight Japanese RVCs were assigned into single genotype (i.e., G4, P[2], I2, R2, C2, A2, N2, T2, E2 and H2 for VP7, VP4, VP6, VP1, VP2, NSP1, NSP2, NSP3, NSP4 and NSP5 genes, respectively). For the VP3 genes, seven Japanese RVCs (HO-61, HO-62, HO-63, HO-64, HO-65, MI-49 and YA-27) were assigned to genotype M3, and one strain, OS-270, was assigned to genotype M3 (Table 2).

To determine the genetic relationship among Japanese RVCs and those detected during the last 27 years (1988–2014) from around the world, phylogenetic trees were constructed for each of the 11 genomic segments of eight Japanese RVCs as well as sequences collected from the GenBank database. Nucleotide positions 49–1047, 21–2258, 23–1210, 12–3284, 37–1691, 55–2136, 38–1222, 43–981, 25–1233, 39–491, and 20–658 were used to compare the VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4 and NSP5 trees, respectively. The human RVCs appear to be distinct from bovine, porcine and canine RVCs (Supplement 2), as reported (Baek et al., 2013; Chen et al., 2002; Marton et al., 2015a; Marton et al., 2015b; Mawatari et al., 2014; Soma et al., 2013; Yamamoto et al., 2011). Among human RVCs, a lineage or sub-lineage was defined as a cluster of sequences having a bootstrap support of greater than 70% at a branching point. However, strict application of this criterion had to be abandoned for the NSP4 gene because there were not enough informative sites due to the shortness of the gene.

In the VP7 phylogenetic tree, within the human RVCs, five lineages (designated lineage I to V) were identified, according to our definition of a lineage as a collection of sequences in a cluster with bootstrap supports >70% (Fig. 1). The first lineage (I) contained two human RVCs from Japan that were detected in 1989–1990. The second lineage (II) contained 13 human RVCs detected between 1988 and 2009 in European countries (United Kingdom, Sweden and Spain), Argentina and Russia. The third lineage (III) contained RVCs from India, Bangladesh, Spain and Nigeria found in 1997–2012. This lineage III was reported to have different genetic backgrounds from other RVCs detected in Far East Asian countries (Yamamoto et al., 2011). Interestingly, however, in this study, we found one RVC, OS-270, detected in Osaka prefecture, Japan in 2012, belonged to this lineage III. In addition, another Far East Asian strain, CAU10-312 detected in South Korea in 2010, also belonged to lineage III. The fourth lineage (IV) contained nine RVCs detected in Japan and Australia in 1988–1995. The last lineage (V) contained 48 RVCs from the Far East Asian, and European countries and Australia between 1992 and 2014. Within lineage V, a further three sub-lineages were identified with sufficiently high bootstrap values of 92–98%: sub-lineage V-1, V-2 and V-3. Sub-lineage V-1 contained 17 VP7 RVC sequences from Japan, Argentina and Sweden between 1996 and 2008, a small sub-lineage of V-2 that contained three VP7 RVC sequences from Japan, China and Australia detected in 1992 and 1995, and sub-lineage V-3 that contained 28 RVC sequences from Japan, Thailand, China, South Korea, Turkey, Colombia and Hungary detected in 1998–2014. Five Japanese RVC sequences, HO-61, HO-62, HO-63, HO-64 and HI-49, detected between 2005 and 2008 in this study clustered into sub-lineage V-1. Other two Japanese strains, HO-65 and YA-27, detected in 2011 clustered into sub-lineage V-3 (Fig. 1).

Similarly, in the nine genome segments (i.e., VP1, VP2, VP4, VP6, NSP1, NSP2, NSP3, NSP4 and NSP5), the Japanese RVCs, along with human RVCs from elsewhere in the world, were largely grouped into two major lineages and two sub-lineages (II, III, V-1 and V-3) (Fig. 2a–b, d–k) in precise correspondence to lineages II and III and sub-lineages V-1 and V-3 designations described for the VP7 phylogenetic tree (Table 3). Due to a lack of whole-genome sequence deposition in the DNA databases of RVCs for lineages I and V and sub-lineage V-2 of VP7 trees, we can't determine their lineages and sub-lineages in the VP1–VP4, VP6 and NSP1–NSP5 trees (Fig. 2a–k). In the VP4, VP6, NSP1 and NSP4 trees, group IV divided into two independent lineages IV-1 and IV-2 (Fig. 2d, e, f and i). Furthermore, the VP4 tree contained one additional lineage VI (Fig. 2d). Lineage VI, comprising the VP4 sequences of more than half of the RVC sequences belonging to sub-lineage V-3 in the other genome segments (Fig. 2d and Table 3). In addition, one independent group (Ex) in the NSP4 tree was completely different from those of E2 genotype strains. The Ex group contains only five Brazilian RVCs detected in 2003–2004 and lacks information on the sequences of the other genome segments.

In the VP3 phylogenetic tree, a different lineage designation was used due to the presence of two genotypes M2 and M3 among human RVCs: lineage I-M3, II-M3, II-M2 and III-M2 corresponded to lineages II and III and sub-lineages V-1 and V-3 of VP7 phylogenetic tree (Fig.2c).

The Indian–Bangladeshi RVCs appear to be distinct from the Far East Asian strains in all 11 genome segments (Fig. 1 and Fig. 2a–k). However, the Japanese OS-270 strain and the South Korean CAU10-312 strain belonged to the Indian–Bangladeshi RVC lineage (lineage III) in all 11 genome segments (Table 3, Fig. 1 and Fig. 2a–k). The nucleotide identities between OS-270, South Korean CAU10-312 and Indian–Bangladeshi RVCs for VP3 ranged from 90.94 to 98.34% and for remaining 10 gene segments ranged from 96.13 to 100% (Table 1).

From the phylogenetic analysis, a circulation of three lineage constellations for contemporary RVCs was observed according to geographic regions. The first lineage constellation of the RVCs in Far East Asian and European countries belonged to the sub-lineage V-1 and V-3 in the all genome segments (I-M3 and II-M3 in the case of the VP3 gene) with an exception of lineage VI in the VP4 gene (Table 3). The second lineage constellation in the Indian–Bangladeshi countries belonged to lineage III in the VP7, VP1-VP3, NSP2, NSP3 and NSP5 or lineage III-1 and III-2 in the VP4, VP6, NSP1 and NSP4 (Table 3). The third lineage constellation is in Russia in which all genome segments belonged to lineage II (II-M2 in the case of the VP3 gene) (Table 3).

The Bayesian evolutionary analysis was used to estimate the evolutionary speed of the 11 genome segments of human RVCs over 27 years (1988–2014). The mean evolutionary rate for all 11 genes

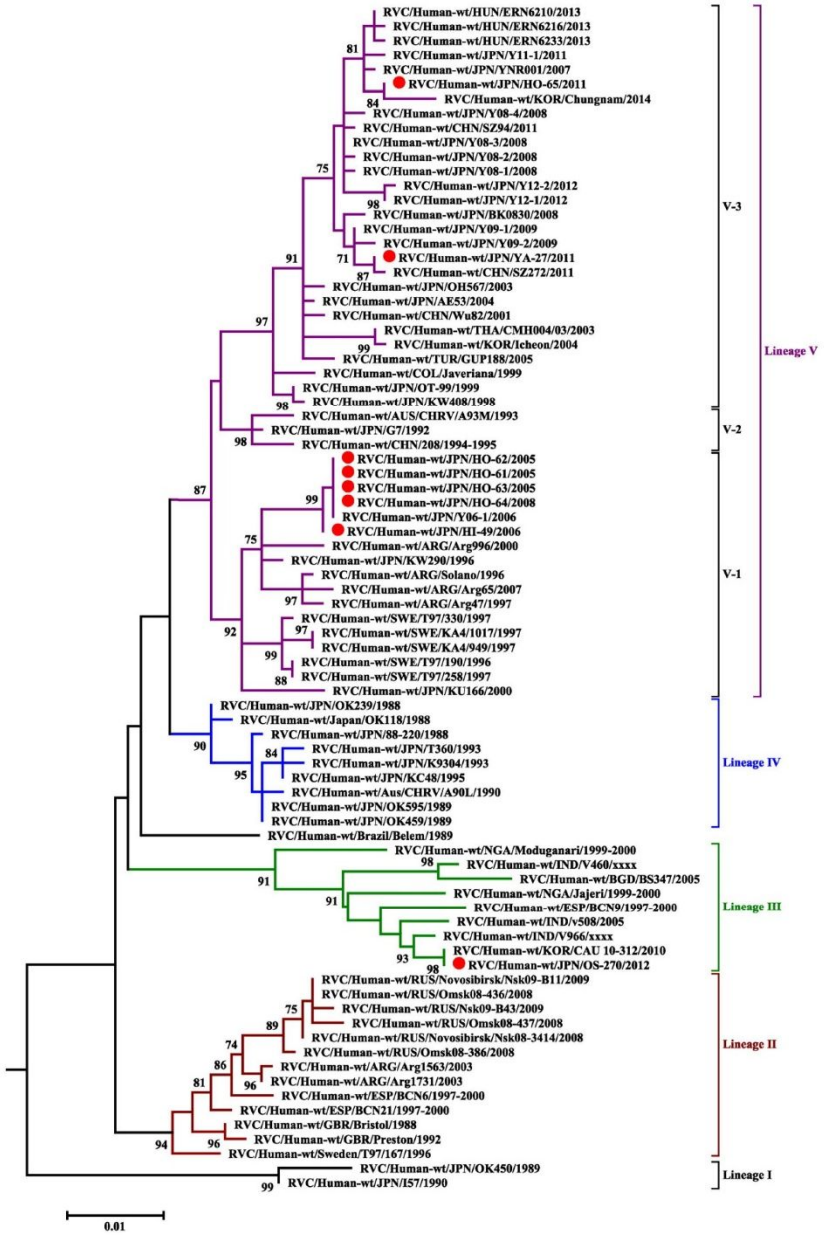


**Fig. 1.** Phylogenetic analyses included the nucleotide VP7 sequences of the eight Japanese RVCs in this study (indicated by red dots) and 82 other RVCs for which VP7 gene was available in the GenBank database. The tree was constructed using the maximum likelihood method that is included in the MEGA6 software package with bootstrap values after 1000 replicate trials. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger. The branch lines and names of lineages I, II, III, IV and V are highlighted in black, maroon, green, blue and purple, respectively. For the sake of space and clearer presentation, only human RVC lineages were presented in figure. All of human RVCs carried genotype G4 for VP7 gene.

ranged from 5.33E−04 to 1.28E−03 substitutions/site/year. Although the 95% highest posterior density (HPD) of all the rates overlapped, the evolutionary rate among the 11 gene segments differed significantly ($p < 0.001$, Kruskal–Wallis test). The mean rates of nucleotide substitutions/site/year were the lowest for NSP2 (5.33E−04; HPD: 3.050E−4 to 7.726E−4) and the highest for NSP4 (1.28E−03; HPD: 9.162E−4 to 1.693E−3). The mean rates of nucleotide substitutions/site/year for remaining nine genome segments are shown in Table 4.

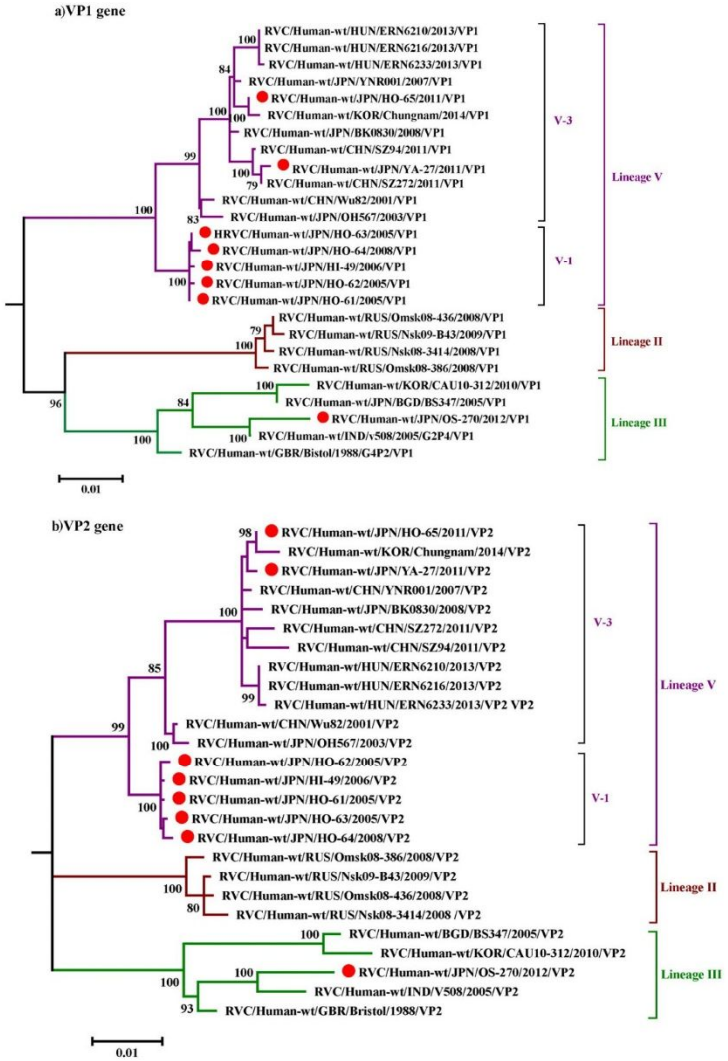


**Fig. 2.** Phylogenetic trees were constructed for the genes encoding (a) VP1, (b) VP2, (c) VP3, (d) VP4, (e) VP6, (f) NSP1, (g) NSP2, (h) NSP3, (i) NSP4 and (k) NSP5. Phylogenetic analysis included the nucleotide sequences of the eight Japanese RVCs in this study (indicated by red dots) and 27 other human and animal G2P[4] strains for which all 11 genome segments were available in the GenBank database. In addition, the 21, 20 and 42 sequences for VP4, VP6 and NSP4 genes of human RVCs were added in the analyses, respectively. The trees were constructed using the maximum likelihood method included in the MEGA6 software package with bootstrap values after 1000 replicate trials. The genetic distance is indicated at the bottom. Percent bootstrap support is indicated by the value at each node when the value was 70% or larger. For the sake of space and clearer presentation, only human RVC lineages were presented in figure. In the VP1–VP4, VP6 and NSP1–NSP5 genes (Fig. 2a–k), the branch lines and names of lineage are also highlighted in the same colors used for the tree of the VP7 genes.

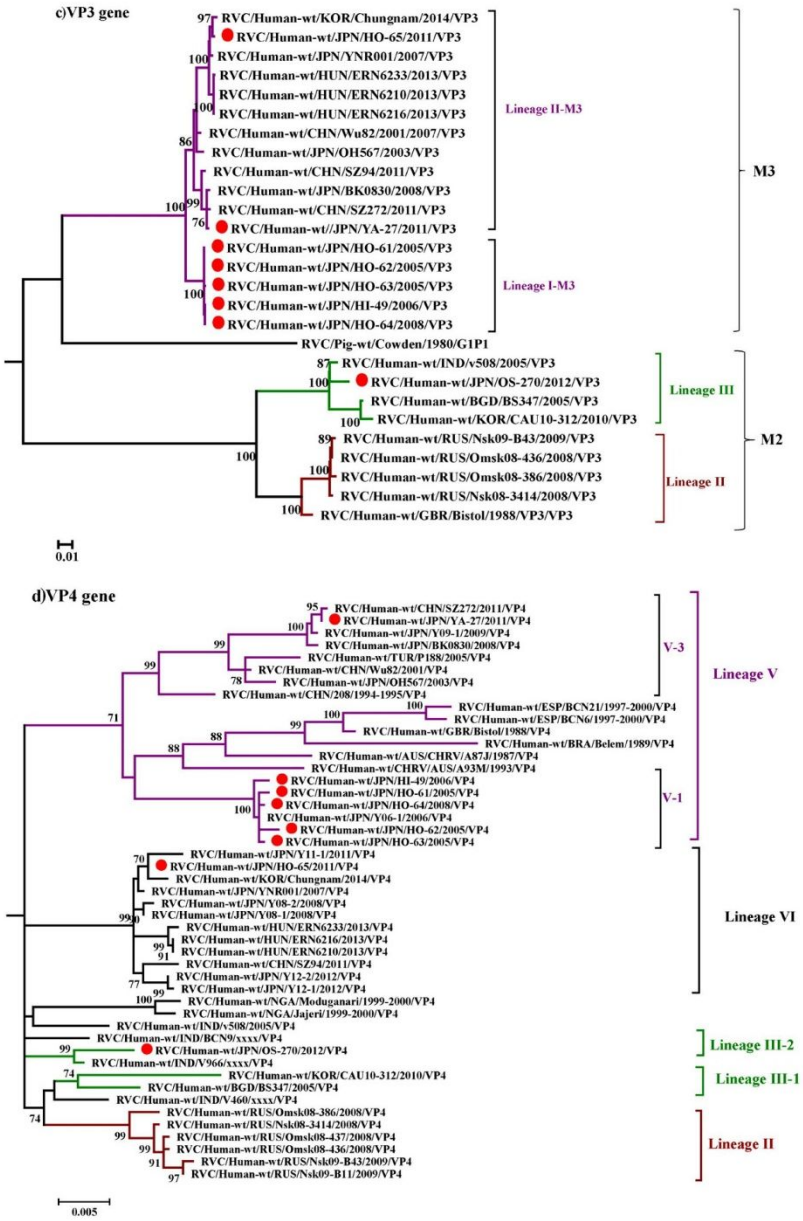


Fig. 2 (*continued*).

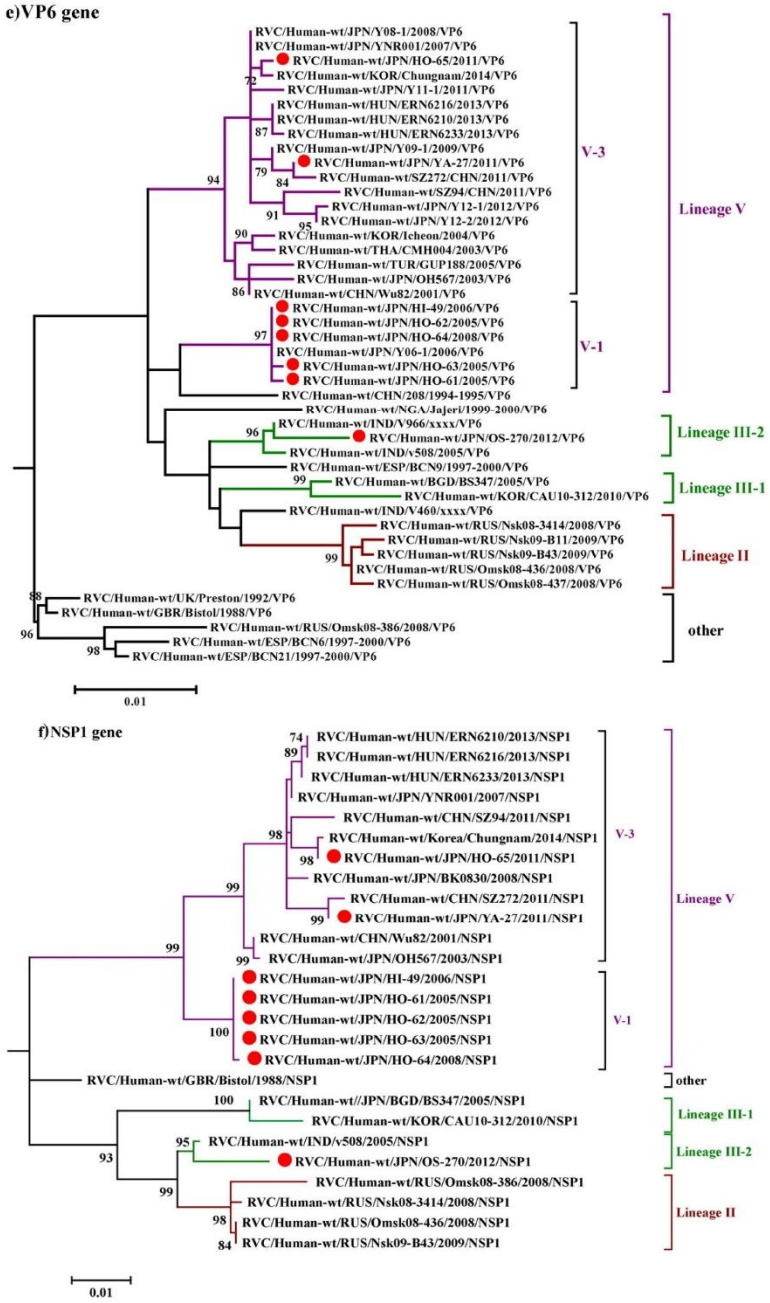


**Fig. 2** (*continued*).

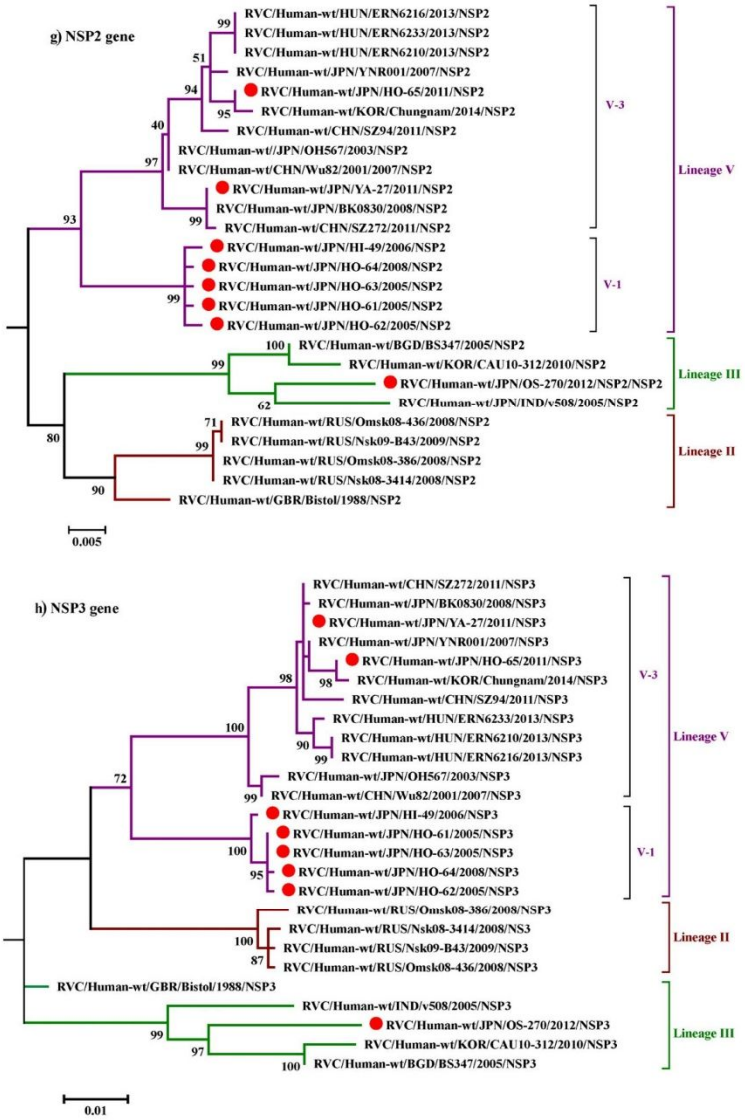


**Fig. 2** (*continued*).

## 4. Discussion

At the genotype level, the eight newly identified Japanese RVCs in this study, together with global human RVCs, were assigned into a single genotype for nine genome segments (i.e., G4, P[2], I2, R2, C2, A2, N2, T2 and H2) for VP7, VP4, VP6, VP1, VP2, NSP1, NSP2, NSP3 and NSP5, respectively). For the VP3 gene, human RVCs were assigned into two genotypes M2 and M3 as reported (Baek et al., 2013; Yamamoto et al., 2011). For the NSP4 gene, most of human RVCs were assigned into single genotype E2, except the five Brazilian RVCs detected in 2003–2004. The nucleotide identity between the Ex group and E2 genotype strains (65.17–68.7%) is lower than the cut-off value of genotypes for NSP4

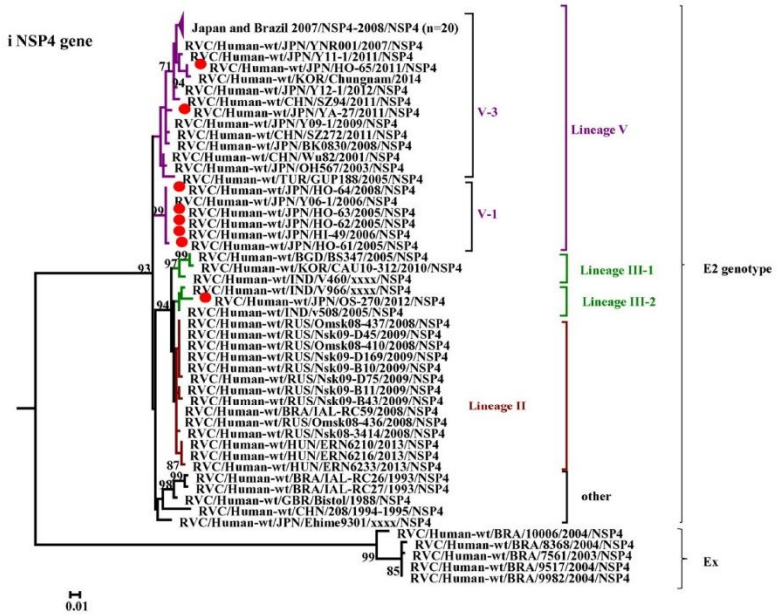


**Fig. 2** (*continued*).

**Table 3**
The lineage constellation of 8 newly determined Japanese RVCs as well as those of 18 human RVCs for which all 11 genome segments were available in the GenBank databases.

| | Strain names | Country of isolation | Year of isolation | VP7 | VP4 | VP6 | VP1 | VP2 | VP3 | NSP1 | NSP2 | NSP3 | NSP4 | NSP5 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human | Wu82 | China | 2001 | V–3 | V–3 | V–3 | V–3 | V–3 | II–M3 | V–3 | V–3 | V–3 | V–3 | V–3 | In circulation |
| Human | OH567 | Japan | 2003 | V–3 | V–3 | V–3 | V–3 | V–3 | II–M3 | V–3 | V–3 | V–3 | V–3 | V–3 | |
| Human | YNR001 | Japan | 2007 | V–3 | VI | V–3 | V–3 | V–3 | II–M3 | V–3 | V–3 | V–3 | V–3 | V–3 | |
| Human | BK0830 | Japan | 2008 | V–3 | V–3 | V–3 | V–3 | V–3 | II–M3 | V–3 | V–3 | V–3 | V–3 | V–3 | |
| Human | HO–65 | Japan | 2011 | V–3 | VI | V–3 | V–3 | V–3 | II–M3 | V–3 | V–3 | V–3 | V–3 | V–3 | |
| Human | YA–27 | Japan | 2011 | V–3 | V–3 | V–3 | V–3 | V–3 | II–M3 | V–3 | V–3 | V–3 | V–3 | V–3 | |
| Human | SZ272 | China | 2011 | V–3 | V–3 | V–3 | V–3 | V–3 | II–M3 | V–3 | V–3 | V–3 | V–3 | V–3 | |
| Human | SZ94 | China | 2011 | V–3 | VI | V–3 | V–3 | V–3 | II–M3 | V–3 | V–3 | V–3 | V–3 | V–3 | |
| Human | ERN6210 | Hungary | 2013 | V–3 | VI | V–3 | V–3 | V–3 | II–M3 | V–3 | V–3 | V–3 | II | V–3 | |
| Human | ERN6216 | Hungary | 2013 | V–3 | VI | V–3 | V–3 | V–3 | II–M3 | V–3 | V–3 | V–3 | II | V–3 | |
| Human | ERN6233 | Hungary | 2013 | V–3 | VI | V–3 | V–3 | V–3 | II–M3 | V–3 | V–3 | V–3 | II | V–3 | |
| Human | Chungnam | South Korea | 2014 | V–3 | VI | V–3 | V–3 | V–3 | II–M3 | V–3 | V–3 | V–3 | V–3 | V–3 | |
| Human | 208 | China | 1994–1995 | V–2 | – | – | – | – | – | – | – | – | – | – | |
| Human | A93M | Australia | 1993 | V–2 | – | – | – | – | – | – | – | – | – | – | |
| Human | G7 | Japan | 1992 | V–2 | – | – | – | – | – | – | – | – | – | – | |
| Human | HI–49 | Japan | 2006 | V–1 | V–1 | V–1 | V–1 | V–1 | I–M3 | V–1 | V–1 | V–1 | V–1 | V–1 | |
| Human | HO–61 | Japan | 2005 | V–1 | V–1 | V–1 | V–1 | V–1 | I–M3 | V–1 | V–1 | V–1 | V–1 | V–1 | |
| Human | HO–62 | Japan | 2005 | V–1 | V–1 | V–1 | V–1 | V–1 | I–M3 | V–1 | V–1 | V–1 | V–1 | V–1 | |
| Human | HO–63 | Japan | 2005 | V–1 | V–1 | V–1 | V–1 | V–1 | I–M3 | V–1 | V–1 | V–1 | V–1 | V–1 | |
| Human | Y06–1 | Japan | 2006 | V–1 | V–1 | V–1 | – | – | – | – | – | – | V–1 | – | |
| Human | HO–64 | Japan | 2008 | V–1 | V–1 | V–1 | V–1 | V–1 | I–M3 | V–1 | V–1 | V–1 | V–1 | V–1 | |
| Human | OK239 | Japan | 1988 | IV | – | – | – | – | – | – | – | – | – | – | Extinct |
| Human | OK459 | Japan | 1989 | IV | – | – | – | – | – | – | – | – | – | – | |
| Human | A90L | Australia | 1990 | IV | – | – | – | – | – | – | – | – | – | – | |
| Human | T360 | Japan | 1993 | IV | – | – | – | – | – | – | – | – | – | – | |
| Human | KC48 | Japan | 1995 | IV | – | – | – | – | – | – | – | – | – | – | |
| Human | v508 | India | 2005 | III | other | III–2 | III | III | III–M2 | III–2 | III | III | III–2 | III | In circulation |
| Human | BS347 | Bangladesh | 2005 | III | III–1 | III–1 | III | III | III–M2 | III–1 | III | III | III–1 | III | |
| Human | CAU 10–312 | South Korea | 2010 | III | III–1 | III–1 | III | III | III–M2 | III–1 | III | III | III–1 | III | |
| Human | OS–270 | Japan | 2012 | III | III–2 | III–2 | III | III | III–M2 | III–2 | III | III | III–2 | III | |
| Human | Omsk08–386 | Russia | 2008 | II | II | other | II | II | II–M2 | II | II | II | II | II | In circulation |
| Human | Nsk08–3414 | Russia | 2008 | II | II | II | II | II | II–M2 | II | II | II | II | II | |
| Human | Omsk08–436 | Russia | 2008 | II | II | II | II | II | II–M2 | II | II | II | II | II | |
| Human | Nsk09–B43 | Russia | 2009 | II | II | II | II | II | II–M2 | II | II | II | II | II | |
| Human | Bistol | United Kingdom | 1988 | II | V | other | III | III | II–M2 | other | II | III | other | II | Extinct |
| Human | OK450 | Japan | 1989 | I | – | – | – | – | – | – | – | – | – | – | Extinct |
| Human | 157 | Japan | 1990 | I | – | – | – | – | – | – | – | – | – | – | |

RVCs determined in this study: name in red.
In addition, human RVCs belonging to lineage I, IV and sub-lineage V-2 in the VP7 tree were also included.

(71%). The nucleotide identity for NSP4 gene between the Ex group and bovine, porcine and canine RVCs (41.37 to 48.63%) are also lower than the cut off value (71%). Thus, the five Brazilian RVCs belonging to the Ex group could be assigned to novel genotype for NSP4 gene. Due to the lack of sequence information for other genome segments of these five Brazilian RVCs, we are not able to determine the genotype for other genes. Overall, all human RVCs might have the same backbone with some VP3 and NSP4 genes inserted into this common backbone.

At the phylogenetic lineage level, this study revealed the prolonged circulation of three lineage constellations (II, III and V) and the possible extinction of two others (I and IV) among human RVCs. The genetic background of RVCs seems to be distinct among different geographic areas. However, there were some surprises. We found one Japanese RVC, OS-270 detected in 2012, that carried the genetic background of Indian–Bangladeshi strains. Moreover, Marton et al. (2015a) reported the occurrence of human RVCs in Hungary carrying the Indian and Bangladeshi NSP4 gene on the Far East Asian strain genetic background. In addition, Baek et al. (2013) also reported one South Korean RVC detected in 2010 that carried the genetic background of Indian–Bangladeshi strains. These observations indicated that, although there are differences of genetic background according to geographical regions, the RVCs were beginning to spread from one region to another.

To our knowledge, this is the first report of the appearance of Indian–Bangladeshi RVC in Japan. We determined the full genome sequence of the other eight human RVCs in Japan between 2005 and 2012, bringing the total number of Far East Asian RVCs that have complete genome sequences to 16. Among these 16 RVCs, interestingly, one Japanese RVC OS-270 and one South Korean RVC were found to have Indian–Bangladeshi lineage constellation, and the remaining 14 (88%) strains carried the typical East Asian and European lineage constellation. OS-270 was collected from a 6-year-old student in a diarrheal outbreak at an elementary school in Osaka, Japan, in 2012. The history of

**Table 4**
The evolutionary rates and 95% HPD (confidence interval) for 11 genome segments of human RVCs over 27 years (1988–2014).

| | n | Sampling time interval | Evolutionary rates (nucleotide substitutions/site/year) | |
|---|---|---|---|---|
| | | | Average | 95% HPD interval |
| VP7 | 81 | 1988–2014 | 7.39E−04 | [5.766E−4, 9.040E−4] |
| VP4 | 53 | 1988–2014 | 7.55E−04 | [5.945E−4, 9.308E−4] |
| VP6 | 46 | 1988–2014 | 6.63E−04 | [5.001E−4, 8.431E−4] |
| VP1 | 26 | 1988–2014 | 7.20E−04 | [5.808E−4, 8.706E−4] |
| VP2 | 26 | 1988–2014 | 9.47E−04 | [7.704E−4, 1.134E−3] |
| VP3 | 26 | 1988–2014 | 7.13E−04 | [4.916E−4, 9.646E−4] |
| NSP1 | 26 | 1988–2014 | 9.50E−04 | [6.788E−4, 1.239E−3] |
| NSP2 | 26 | 1988–2014 | 5.33E−04 | [3.050E−4, 7.726E−4] |
| NSP3 | 26 | 1988–2014 | 9.92E−04 | [6.739E−4, 1.327E−3] |
| NSP4 | 68 | 1988–2014 | 1.28E−03 | [9.162E−4, 1.693E−3] |
| NSP5 | 26 | 1988–2014 | 5.79E−04 | [3.486E−4, 8.655E−4] |

travel in Indian–Bangladeshi countries in the period before and during this outbreak of diarrheal students or their caregivers was not known. However, together with the appearance of Indian–Bangladeshi RVC in South Korea in 2010, this study indicated that the Indian–Bangladeshi RVC might have been existing in the human population of Far East Asian countries. It was not clear when Indian–Bangladeshi RVC came to Japan and whether this strain could spread with high prevalence in Japan and other Far East Asian countries or not. Therefore, the continued surveillance of RVCs is also needed to evaluate RVC diversity in different geographical regions.

When the Japanese RVCs in this study were examined in the context of molecular epidemiology of other Far East Asian RVCs deposited in GenBank databases, we observed that the contemporary Far East Asian RVCs in lineage V may have originated from strains in lineage IV around 1987 (Fig. 3). Within the contemporary Far East Asian strains (lineage V), diversification of RVCs further led two sub-lineages V-1 and V-3 in all 11 genes, and there was the sub-lineage constellation shift from V-1 to V-3. The lineage shifts have been also documented for RVAs, such as human G2P[4] strains (Doan et al., 2015; Doan et al., 2011).

The intra-genotype and inter-genotype reassortments among human rotaviruses or between human and animal rotaviruses associated with interspecies transmission have been well understood for RVAs (Ghosh and Kobayashi, 2014). In terms of RVCs, the intra-genotype reassortment events also were observed among human RVCs in this study and a study in Hungary (Marton et al., 2015a). The inter-genotype reassortment events have not been found in the human RVCs because one genotype has been reported for each genome

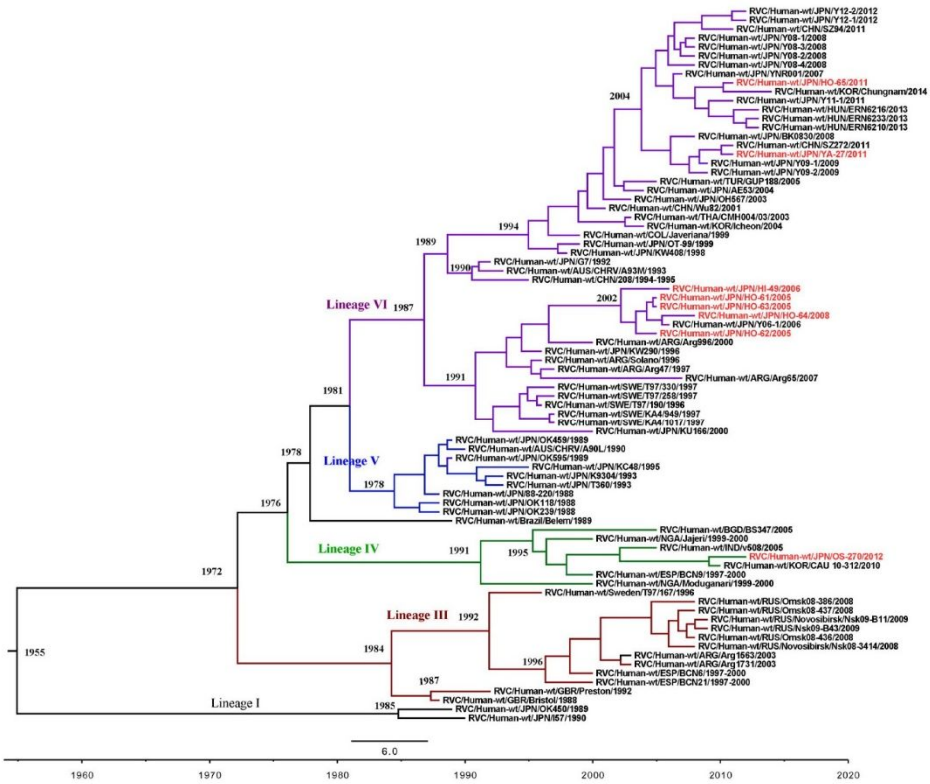


**Fig. 3.** Maximum clade credibility (MCC) tree for the VP7 genes of 80 human RVCs detected from 1988 to 2014. MCC tree was constructed using the Bayesian MCMC framework. The years of divergence of each lineage are indicated at each node.

segment of human RVCs, except the VP3 gene which had 2 genotypes M2 and M3. Although porcine RVCs showed at least seven VP7 genotypes (G1, G3, G5, G6, G7, G8 and G7) (Marthaler et al., 2013), but the whole genome information for porcine RVCs is limited to one strain only, Cowden isolated in 1980. Therefore the inter-genotype reassortment events among porcine RVCs also have not been reported in previous studies.

With regard to the interspecies transmission, there is no evidence of animal RVC introduction to the human RVCs in this study. In a previous study, however, Gabbay et al. (2008) reported the evidence of interspecies transmission of RVCs from a pig to a human in Brazil, where both human and porcine RVCs were endemic (Gabbay et al., 2008). Only the VP6 gene of human strains was characterized in their analysis, therefore it is not clear whether the interspecies transmission of RVC from pig to human was a direct transmission event as a whole genome constellation or gene segment reassortment between human and porcine RVCs.

The evolutionary rates obtained for human RVC VP7 genes (7.39E−04 substitutions/site/year) was found to be lower than that of human RVA VP7 genes such as human G2 genotype (0.95E−03 substitutions/site/year) (Doan et al., 2015). Similar, the mean rates of nucleotide substitutions/site/year of human RVC for VP4 (7.55E−04) and NSP2 (5.33E−04) were slightly lower as compared with those of human RVA VP4 genes (5.86E−3) and NSP2 genes from genotype N1 (0.87E−03), respectively (Donker and Kirkwood, 2012; Jenkins et al., 2002). Moreover, the evolutionary rate of human RVC VP3 genes from two genotypes (7.13E−04 substitutions/site/year) was also lower than that of human RVA VP3 gene from single genotype M2 (1.11E−03 substitutions per site per year) (Doan et al., 2015). In contrast, the evolutionary rate of NSP4 gene for human RVCs (1.28E−3 substitutions/site/year) was slightly higher than that of E2 NSP4 for human RVA (1.05E−3 substitutions/site/year). The higher evolutionary rate of NSP4 gene as compared to those of other genes of human RVAs may reflect the presence of novel genotype (Ex) for NSP4 gene of human RVCs. When the evolutionary rates of human RVCs (ranged from 5.33E−04 to 1.28E−03) were compared to those of human RVBs (ranged from 1.36E−3 to 4.78E−3) (Jenkins et al., 2002), there were clear statistically significant differences for all genome segments, except NSP4 genes. The evolution rates of human RVCs were lower than those of human RVAs and RVBs reflecting the higher conservation level of RVC genome.

In conclusion, over 27 years (1988–2014) the human RVCs showed the prolonged circulation of three lineage constellations (II, III and V) and the possible extinction of two other lineage constellations (I and IV). This is the first report about the appearance of Indian–Bangladeshi RVC in Japan in 2012. Taken together with the appearance of Indian–Bangladeshi RVC in South Korea in 2010 and the reassortment event between two different lineage constellations in Hungary in 2013, this study indicated that the RVCs have begun their spread from one region to another, although there were differences of genetic background of human RVCs according to geographical regions in the past. Therefore, the continued surveillance of RVCs is also needed to determine RVC diversity in different geographical regions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2016.03.027.

## Conflict of interest

The authors declare that they have no conflict of interest.

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