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Diversity of group A rotavirus genes detected in the Triângulo Mineiro region, Minas Gerais, Brazil

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ABSTRACT

Group A rotaviruses are the main causative agent of infantile gastroenteritis. The segmented nature of the viral genome allows reassortment of genome segments, which can generate genetic variants. In this study, we characterized the diversity of the VP7, VP4 (VP8*), VP6, NSP4, and NSP5 genes of the rotaviruses that circulated from 2005 to 2011 in the Triângulo Mineiro (TM) region of Brazil. Samples with genotypes G2 (sublineages IVa-1 and IVa-3), G1 (sublineage I-A), G9 (lineage III), G12 (lineages II and III), G8 (lineage II), G3 (lineage III), P[4] (sublineages IVa and IVb), P[8] (sublineages P[8]-3.6, P[8]-3.3, and P[8]-3.1), I2 (lineage VII), E2 (lineages VI, XII, and X), and H2 (lineage III) were identified. The associations found in the samples were G1, G9, or G12 with P[8]-I1-E1-H1; G2 or G8 with P[4]-I2-E2-H2; G12 with I3-E3-H6; and G3 with P[4]-I2-E3-H3 (previously unreported combination). Reassortment events in G2P[4] strains and an apparent pattern of temporal segregation within the lineages were observed. Five TM samples contained genes that exhibited high nucleotide and amino acid identities with strains of animal origin. The present study includes a period of pre- and post-introduction of rotavirus vaccination in all Brazilian territories, thereby serving as a basis for monitoring changes in the genetic constitution of rotaviruses. The results also contribute to the understanding of the diversity and evolution of rotaviruses in a global context.

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Introduction

The group A rotavirus (RVA), a member of the genus *Rotavirus* in the family *Reoviridae*, is the main causative agent of severe diarrheal disease in children. In 2008, the virus was associated with the deaths of 453,000 children less than 5 years of age worldwide, with most cases reported in Africa and Asia.[1]

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The viral particle is non-enveloped and composed of 11 segments of double-stranded RNA surrounded by a triple-layered protein capsid. The viral genome encode six structural (VP1–VP4, VP6 and VP7) and six non-structural (NSP1–NSP6) proteins. The outer capsid proteins, VP7 and VP4 (cleaved by trypsin into VP5* and VP8*), are involved in the attachment and entry during viral replication in host cells, and each can independently induce the production of neutralizing antibodies. The serotypes defined by VP7 and VP4, as well as their encoding genes, are used to classify rotaviruses into G and P genotypes, respectively, based on a widely used binomial system.[2] Previous studies have demonstrated that RVAs of genotypes G1, G2, G3, G4, G9, and G12, combined with genotypes P[4] or P[8], are most prevalent among the strains that cause human infection.[3]

Events such as point mutations, rearrangements, interspecies transmissions, and reassortments occurring during co-infections with different strains can generate genetic variants with vast inter- and intragenotypic diversity. This high variability and observed fluctuations in the prevalence of dominant genotypes throughout epidemic seasons represent a major challenge for immunization efforts using vaccines currently available for the prevention of severe infections caused by RVAs in many countries. To better characterize the diversity and evolutionary aspects of rotaviruses, a new classification system based on the 11 segments of the RVA genome has recently been proposed, which emphasizes the importance of analyzing other genes in addition to those that encode for the VP7 and VP4 proteins.[4] In this latter classification, the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6-encoding gene segments are classified as Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (x indicates the number of the genotype), respectively. Genes 6, 10, and 11 encode the VP6, NSP4, and NSP5 proteins, respectively. The VP6 protein has been studied as a possible target for vaccine development, and studies in animals have demonstrated protective immune responses induced by VP6 protein immunization.[5] NSP4 is involved in viral morphogenesis and has enterotoxin-like activity.[6] Last, NSP5 is essential for viroplasm formation, genome replication, and virion assembly, and its gene is most frequently associated with rearrangement events.[2]

Since 2005, surveillance of circulating RVA strains has been conducted in the Triângulo Mineiro (TM) region of Minas Gerais State, Brazil. The studies showed that different G-types associated with P[8] co-circulated in 2005 and also that G2P[4] had emerged by mid-2006, continuing as the prevalent genotype until 2010.[7–9] In this study, we characterized the diversity of the VP7, VP4 (VP8* subunit), VP6, NSP4, and NSP5 genes of representative RVA strains that circulated during six seasons (2005–2011) in the TM region.

Materials and methods

Sample collection

Twenty-seven representative RVA strains from a collection of 117 rotavirus-positive fecal samples from Uberaba (2005–2009 and 2011) and Uberlândia (2008–2011) were selected based on their electropherotypes and VP7 and VP4 genotypes, previously characterized in three-laboratory-based surveillance studies (Table 1).[7–9] These previous studies analyzed fecal specimens from children who presented acute gastroenteritis, which were provided by laboratories Jorge Furtado Medicina Diagnóstica (Uberaba) or IPAC Medicina Diagnóstica (Uberlândia). Information on the vaccination history of the patients was not available. The 27 representative samples were collected from outpatients and inpatients from one hospital in Uberaba and two hospitals in Uberlândia. This study was approved by the Human Research Ethics Committee of Universidade Federal do Triângulo Mineiro (protocol CEP/UFTM/672/06).

RNA extraction, reverse transcription polymerase chain reaction (RT-PCR), and nucleotide (nt) sequencing

The fecal specimens were subjected to nucleic acid extraction,[10] and reverse transcription was carried out using random primers (Invitrogen, Carlsbad, CA, USA) and

Table 1 – Selected RVA G/P types collected from children with gastroenteritis in Uberaba and Uberlândia, Triângulo Mineiro, Brazil, from 2005 to 2011.

G/P type	City	2005–2006	2007	2008	2009	2010	2011
		Collected/ selected	Collected/ selected	Collected/ selected	Collected/ selected	Collected/ selected	Collected/ selected
G1P[8]	UB	3/1	–	–	–	–	–
G2P[4]	UB	25/4	33/3	5/2	–	–	–
	IP	–	–	19/3	–	16/3	–
G9P[8]	UB	7/4	–	–	–	–	–
G3P[4][a]	IP	–	–	–	–	–	1/1
GNTP[8]	UB	2/2	–	–	–	–	–
G3P[NT]	IP	–	–	–	1/1	–	–
GNTP[NT]	UB	2/2	–	–	–	–	1/1
Mixed	UB	–	–	–	1/0	–	–
	IP	–	–	1/0	–	–	–
Total		39/13	33/3	25/5	2/1	16/3	2/2

UB, Uberaba; IP, Uberlândia; NT, non-typed.

[a] Typed as G3 by PCR but determined to be G8 by sequence analysis.

murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturers' instructions. The complementary DNA generated by the RT reaction was then used for the amplification of all genes. The primers and reaction conditions for amplification of each target gene were based on previously described protocols as follows: primers VP7-F and VP7-R for partial (nt positions 49 to 933) VP7 gene[11]; VP4-F and VP4-R for the VP8* subunit (nt positions 132 to 795) of the VP4 gene[12]; JRG30(+) and JRG31(−) for a full-length gene (nt positions 1 to 738) encoding the NSP4 protein[13]; VP6-F and VP6-R for a partial gene (nt positions 747 to 1126) encoding the VP6 protein[14]; and GEN-NSP5-F and GEN-NSP5-R for a full-length gene (nt positions 1 to 667 or to 1043) encoding the NSP5 protein.[15] PCR products were purified using the EasyPrep Gel/PCR Purification Mini Kit (EasyPath, São Paulo, SP, Brazil), separated by agarose gel electrophoresis, and then quantified by comparing the band intensities with those of the Low DNA Mass Ladder (Invitrogen, Carlsbad, CA, USA). The DNA fragments were then sequenced in both directions with the same primers as used for the PCRs using the BigDye® Terminator Cycle Sequencing Kit v. 3.1 and an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Genotype assignment and phylogenetic analysis

The VP7, VP8*, VP6, NSP4, and NSP5 gene sequences were curated with the Geneious software (Biomatters Ltd., Auckland, New Zealand). To identify the genotypes and evaluate the genetic diversity, the TM samples were compared with the reference strains representing distinct genotypes, lineages, and sublineages and with most similar field samples from different continents, as well as with vaccine strains. The lineages and sublineages of G2, P[4], I2, E2, and H2 samples were previously suggested by Giammanco et al.,[16] whereas the G1, G9, G12, G8, G3, and P[8] lineages and sublineages were described by Phan et al.,[17,18] Rahman et al.,[19] Ianiro et al.,[20] Ndze et al.,[21] and da Silva et al.,[22] respectively. All sequences of the field and reference strains were retrieved from GenBank. Alignments were performed using MUSCLE (Multiple Sequence Comparison by Log-Expectation) and analyzed with the maximum likelihood algorithm.[23,24] The best-fit model of sequence evolution was estimated by jModelTest according to the Bayesian Information Criterion, and phylogenetic trees were constructed for each gene. The maximum-likelihood trees were generated using selected models of nt substitution, GTR+I+G (VP7), HKY+G (VP8* and NSP5), HKY+G+I (VP6), and TrN+G (NSP4), in the Molecular Evolutionary Genetics Analysis 5.1 software (MEGA Team, Japan). The nomenclature of all rotavirus strains in the trees is listed according to Matthijnssens et al.[25] The UB, IP and MG abbreviations in the nomenclature of the TM samples represent Uberaba, Uberlândia, and Minas Gerais State, respectively.

Nucleotide sequence accession numbers

The nucleotide sequences were deposited in the GenBank database, and the accession numbers are: KF938698–KF938771 for the G2P[4] samples and KR018449–KR018504 for the other G-types. The accession numbers of the strains retrieved from GenBank are shown at the end of each name in the trees.

Results

A total of 130 nt consensus sequences were obtained for the 27 representative TM samples. In four of the samples, at least one of the five target genes was not amplified with the primers used.

Based on the VP7 gene, six genotypes were characterized: G1, G2, G3, G8, G9, and G12 (Fig. 1a). The TM G2 samples shared 95.3–100% and 96.8–100% nt and amino acid (aa) identity, respectively. They belonged to lineage IV, segregating into sublineages IVa-3 and IVa-1. The TM G1 sample belonged to lineage I, sublineage I-A, and shared 94% nt and 95.3% aa identity with the Rotarix™ vaccine strain of lineage II. The TM G9 samples belonged to lineage III and shared 97.3–100% nt and aa identity. The TM G12 samples shared 96.4–100% and 96.7–100% nt and aa identity, respectively. These samples clustered into lineages II (with strains of G12P[9] combination) and III (with strains of G12P[8] and G12P[6] combinations). The TM G8 sample belonged to lineage II; it exhibited high nt (99.3–99.9%) and aa (99.1–100%) identities with other contemporary human P[4] samples from Italy (SS56), USA (USRo104), Germany (GER1H-09), and Brazil (RN19461-08). The TM G3 sample fell into lineage III, in a separate sub-cluster from the prototype strain (AU-1). They only shared 90.7% nt and 94.9% aa identity, but the TM G3 sample showed higher identity (nt, 99.1%; aa, 98.4%) with a cat sample (BA222) isolated from Italy.

Based on the VP8* gene, the TM samples were distributed among P[8]- and P[4]-types (Fig. 1b). The TM P[4] samples shared 96.2–100% and 95.2–100% nt and aa identity, respectively. These samples belonged to lineage IV and split into sublineages IVa and IVb. The TM P[8] samples showed 96.5–100% nt and 95.6–100% aa identity. They belonged to lineage P[8]-3 and were divided between sublineages P[8]-3.1, P[8]-3.3, and P[8]-3.6, regardless of the combinations with the G genotypes.

Based on the VP6 gene, the TM samples belonged to I1, I2, and I3 genotypes (Fig. 1c). The TM I2 strains showed 95.9–100% and 99.1–100% nt and aa identity, respectively, and clustered together with other strains identified within lineage VII worldwide. All I2 strains shared P[4] genotype specificity and formed two main sub-clusters (a and b) with their separation supported by a high bootstrap value (100%). The TM samples that grouped into cluster I3 exhibited G12 specificity and showed high nt (99.4–100%) and aa (100%) identities to the reference sample (T152) of the G12P[9] combination. The TM I1 samples shared an nt identity of 94.7–100% and an aa identity of 98.2–100%. They grouped apart from the prototype (Wa) and vaccine-derived (Rotarix™) strains. The TM I1 samples shared 87.3–100% nt and 95.6–100% aa identity with these reference strains, and the majority shared P[8] genotype specificity.

Based on the NSP4 gene, the TM samples were distributed among the E2, E1, and E3 genotypes (Fig. 1d). The TM E2 samples shared 85.0–100% nt and 94.0–100% aa identity. They were subdivided between the clusters of lineages VI, X, and XII, composed of strains with P[4] specificity. The TM samples of lineage VI formed two phylogenetic sub-clusters (a and b), with their separation supported by a high bootstrap value (99%). TM strains UB-239 and UB-226 displayed high nt (95.7–100%) and aa (97.7–100%) identities with the bovine 1603

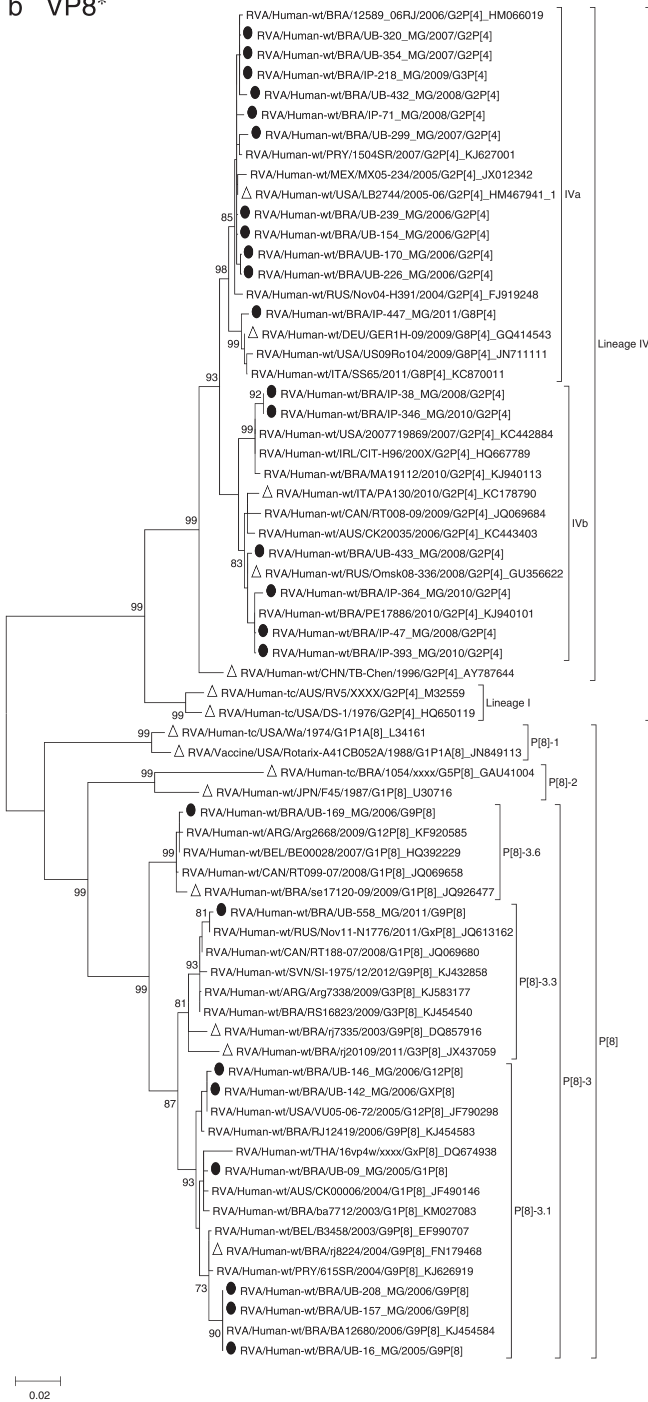
a VP7

RVA/Human-wt/BRA/UB-320_MG/2007/G2P[4]
RVA/Human-wt/BRA/UB-354_MG/2007/G2P[4]
RVA/Human-wt/BRA/UB-432_MG/2008/G2P[4]
RVA/Human-wt/BGD/Dhaka26-06/2006/G2P[4]_EF690795
RVA/Human-wt/BRA/RJ12398/2006/G2P[4]_KJ638611
RVA/Human-wt/ECU/2012826190/2011/G2P[4]_KC951945
RVA/Human-wt/BRA/IP-71_MG/2008/G2P[4]
RVA/Human-wt/BRA/IP-346_MG/2010/G2P[4]
RVA/Human-wt/USA/2007719869/2007/G2P[4]_KC442886
RVA/Human-wt/BRA/IP-38_MG/2008/G2P[4]
RVA/Human-wt/BRA/RJ17745/2010/G2P[4]_KJ638629
RVA/Human-wt/AUS/CK20024/2006/G2P[4]_KC443702
RVA/Human-wt/ITA/PA83/2007/G2P[4]_KC178812
RVA/Human-wt/ARG/Arg2852/2010/G2P[4]_KF920559
RVA/Human-wt/BRA/IP-47_MG/2008/G2P[4]
RVA/Human-wt/BRA/UB-433_MG/2008/G2P[4]
RVA/Human-wt/BRA/IP-393_MG/2010/G2P[4]
RVA/Human-wt/BRA/BA17990/2010/G2P[4]_KJ638630
RVA/Human-wt/CAN/RT008-09/2009/G2P4_JQ069521
RVA/Human-wt/JPN/01P017/2001/G2P[4]_AB821335
RVA/Human-wt/BRA/UB-299_MG/2007/G2P[4]
RVA/Human-wt/BRA/IP-364_MG/2010/G2P[4]
RVA/Human-wt/BRA/UB-226_MG/2006/G2P[4]
RVA/Human-wt/BRA/UB-239_MG/2006/G2P[4]
RVA/Human-wt/ARG/Arg2389/2007/G2P[4]_GQ154537
RVA/Human-wt/BRA/MT11258/2005/G2P[4]_KJ638603
RVA/Human-wt/BRA/UB-154_MG/2006/G2P[4]
RVA/Human-wt/BRA/UB-170_MG/2006/G2P[4]
RVA/Human-wt/USA/LB2772/2005-06/G2P[4]_HM467956
RVA/Human-wt/CHN/Tb-Chen/1996/G2P[4]_AY787646
RVA/Human-wt/AUS/95A/1995/G2PX_HRU73955
RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5]_GU565068
RVA/Human-tc/USA/DS-1/1976/G2P[4]_AB118023
RVA/Human-wt/AUS/Hu5/1977/G2PX_X00572
RVA/Human-tc/JPN/KU/1974/G1P[8]_D16343
RVA/Human-tc/USA/Wa/1974/G1P[8]_JX406755
RVA/Vaccine/USA/Rotarix-A41CB052A/1988/G1P1A[8]_JN849114
RVA/Human-wt/VNM/VN-281/2003/G1Px_DQ508167
RVA/Human-wt/URY/Mvd9816/1996-9/G1Px_AF480293
RVA/Human-tc/CHN/Chi-45/1987/G1Px_GAU26371
RVA/Human-wt/JPN/6916/2005-6/G1P[8]_EF079067
RVA/Human-wt/BGD/Dhaka8-02/xxxx/G1P[8]_AY631049
RVA/Human-wt/AUS/CK00006/2004/G1P[8]_JF490143
RVA/Human-wt/ITA/PR1598/2005/G1P[8]_HM245031
RVA/Human-wt/BRA/ba6599-03/2003/G1P[8]_KM027206
RVA/Human-wt/BRA/UB-09_MG/2005/G1P[8]
RVA/Human-wt/BRA/UB-157_MG/2006/G9P[8]
RVA/Human-wt/BRA/UB-208_MG/2006/G9P[8]
RVA/Human-wt/BRA/BA12680/2006/G9P[8]_KJ454491
RVA/Human-wt/BRA/UB-16_MG/2005/G9P[8]
RVA/Human-wt/BEL/B3458/2003/G9P[8]_EF990708
RVA/Human-wt/JPN/OT-7/2008/G9P[8]_FJ695604
RVA/Human-wt/ARG/Arg2090/2007/G9P[8]_GQ154523
RVA/Human-wt/BRA/ro13270/06/2006/G9P[8]_FJ794015
RVA/Human-wt/BRA/UB-169_MG/2006/G9P[8]
RVA/Human-wt/RUS/RUS-Nov06-1289/2006/G9P[8]_FJ529393
RVA/Human-wt/ITA/MAR37/2006/G9P[8]_EF150339
RVA/Human-wt/DEU/GER96-08/2008/G9P[8]_GU392986
RVA/Human-wt/BRA/UB-558_MG/2011/G9P[8]
RVA/Human-wt/CHN/JS10-15/2012/G9P[8]_KF113010
RVA/Human-tc/JPN/F45/1987/G9P[8]_AB180970
RVA/Human-tc/USA/WI61/1983/G9P[8]_AB180969
RVA/Human-wt/BRA/UB-144_MG/2006/G12Px
RVA/Human-wt/BRA/UB-150_MG/2006/G12Px
RVA/Human-wt/PRY/1471SR/2006/G12P[9]_KJ412580
RVA/Human-tc/THA/T152/1998/G12P[9]_AB071404
RVA/Human-wt/JPN/K12/1999/_G12P[9]_AB186120
RVA/Human-wt/BRA/PE15776/2008/G12P[9]_KF907303
RVA/Human-wt/BGD/N26/2002/G12P[6]_DQ146687
RVA/Human-wt/KOR/CAU195/200x/G12P[6]_EF059916
RVA/Human-wt/GBR/MV404-02/2002/G12P[6]_DQ501280
RVA/Human-wt/USA/US6588/2005/G12P[8]_FJ152110
RVA/Human-wt/BRA/RJ12419/2006/G12P[8]_KF907305
RVA/Human-wt/BRA/UB-146_MG/2006/G12P[8]
RVA/Human-wt/ITA/SS56/2011/G8P[4]_KC870004
RVA/Human-wt/USA/US09Ro104/2009/G8P[4]_JN711112
RVA/Human-wt/DEU/GER1H-09/2009/G8P[4]_GQ414545
RVA/Human-wt/BRA/IP-447_MG/2011/G8P[4]
RVA/Human-wt/BRA/RN19461-08/2008/G8Px_JX844064
RVA/Human-tc/IND/69M/1980/G8P[10]_EF672560
RVA/Human-tc/KEN/B12/1987/G8P[1]_HM627547
RVA/Human-wt/BEL/B4106/2000/G3P[14]_AY740736
RVA/Human-tc/JPN/AU-1/1982/G3P[9]_D86271
RVA/Human-wt/BRA/RS16838/2009/G3P8_KJ454506
RVA/Human-tc/USA/CC425/1997/G3P[9]_AJ311738
RVA/Human-wt/MDG/02.3073/2008-9/G3P[9]_HQ405259
RVA/Human-wt/BRA/IP-218_MG/2009/G3P[4]
RVA/Cat-wt/ITA/BA222/2005/G3P[9]_GU827411
RVA/Human-wt/FRA/R1320/2007/G3Px_HM035513
RVA/Human-wt/RUS/01154/2011/G3P[9]_JX088021

IVa-3 IVa-1 IVa Lineage IV Lineage II Lineage I G2 Lineage III Lineage II I-B I-C I-E I-D I-A Lineage I G1 Lineage III Lineage I G9 Lineage II Lineage III G12 Lineage II Lineage III G8 Lineage I Lineage III G3

0.1

Fig. 1 – Maximum-likelihood phylogenetic analysis based on the VP7 (a), VP8* (b), VP6 (c), NSP4 (d), and NSP5 (e) gene nucleotide sequences of Triângulo Mineiro (TM) strains and the sequences from the GenBank database. The numbers at the nodes indicate bootstrap values (1000 replicates), and only values above 70% are shown. The trees are drawn to scale, with the branch lengths measured as the number of substitutions per site. The TM samples are marked with a solid circle, and the reference strains of each lineage and sublineage are marked with an open triangle. The uppercase letters followed by Arabic numerals, Roman numeral, and lowercase letters represent different genotypes, lineages, and sublineages, respectively, except for lineages and sublineages of the P[8] genotype, which are indicated by Arabic numerals, and sublineages of G1 strains, which are indicated by uppercase letters.



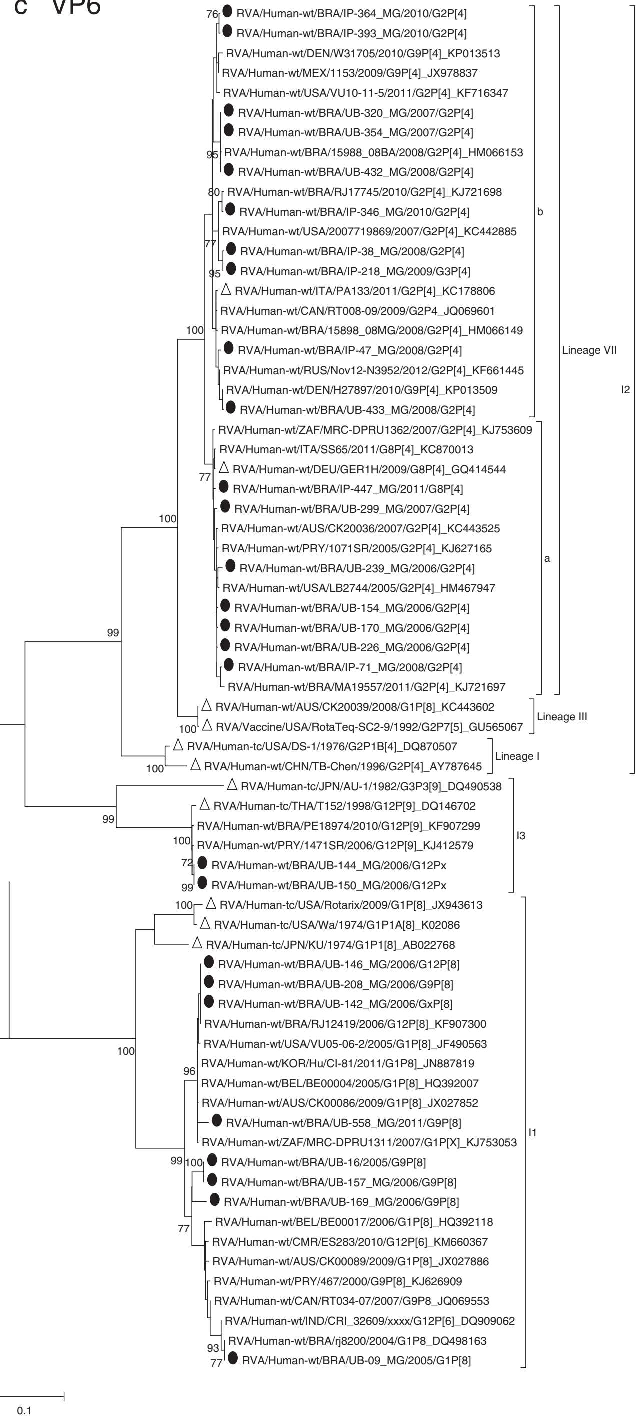


Fig. 1 – (*Continued*)

strain detected in 2007 in South Africa[26] and with a human Brazilian strain, 12389_06RJ (99.0–100% nt and 99.4–100% aa identity), detected in 2006.[27] These four strains did not group with any cluster of the previously established lineages.[16] The TM E1 samples shared 91.7–100% and 92.8–100% nt and aa identity, respectively, and the majority of the strains had the P[8] genotype. The TM E3 samples showed 88.7% and 94.0% nt and aa identity, respectively. They grouped with strains that had genotype P[9] combined with G3, G6, or G12.

Based on the NSP5 gene, the TM samples were distributed among the H1, H6, H3, and H2 genotypes (Fig. 1e). The TM H1 samples shared nt and aa identities in the ranges of 97.3–100% and 99.0–100%, respectively. They grouped with field samples of P[8] specificity and clustered apart from the prototypes, Wa and KU, and the vaccine-derived (Rotarix™) strain. The nt identity with these reference strains was 92.3–100%, and the aa identity was 93.9–100%. The TM H6 samples were identical and clustered together with samples exhibiting the G12P[9]

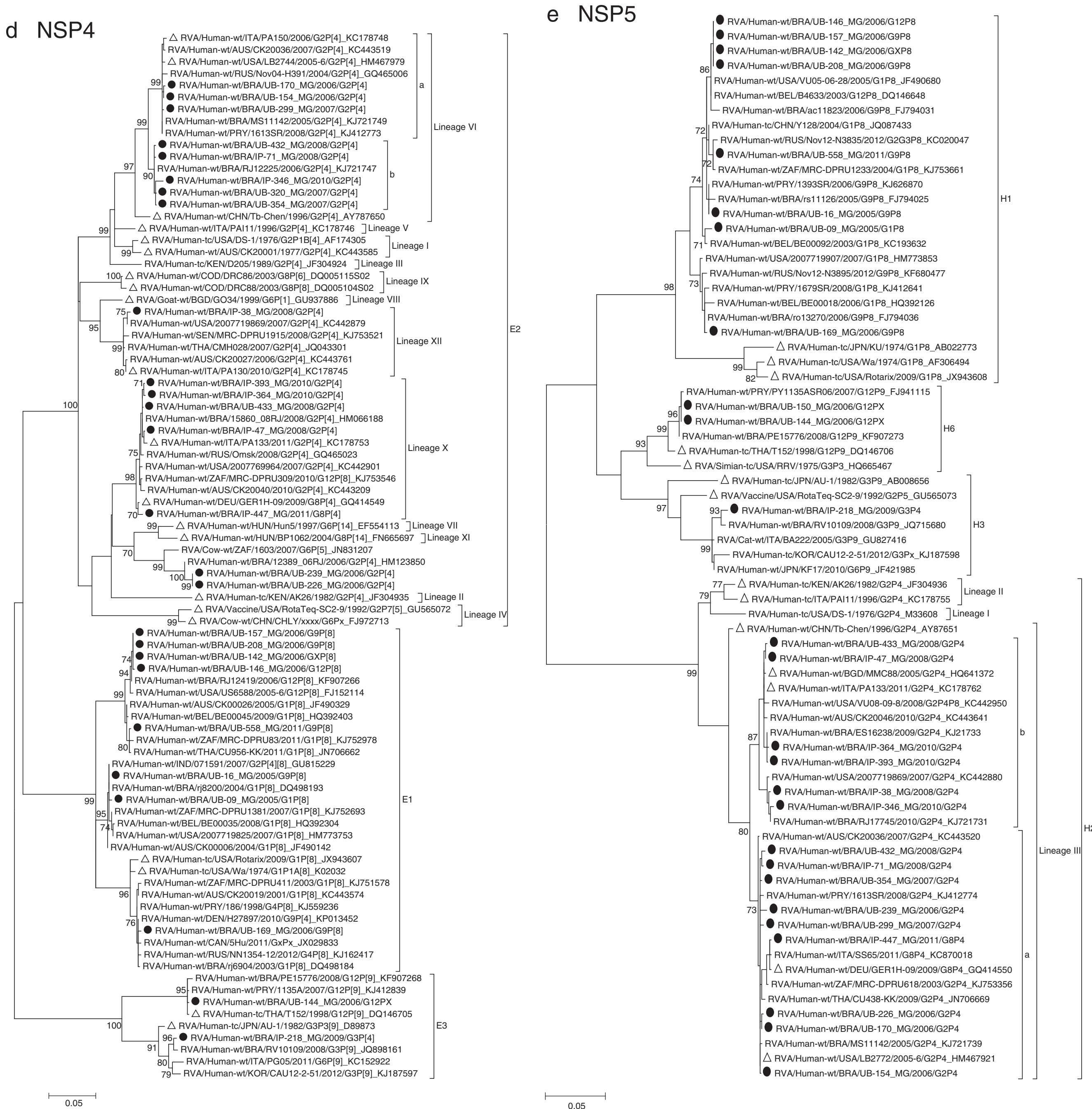


Fig. 1 – ***(Continued).***

combination. They were closely related (nt identity, 94.3%; aa identity, 98.5%) to a simian RVA strain (RRV). The TM H3 sample (IP-218) grouped apart from the prototype (AU-1) and vaccine (RotaTeq^{TM}) strains, with which it showed the nt identity of 93.3–95.2% and the aa identity of 92.9–96.0%. At the same time, sample IP-218 showed high nt (98.7%) and aa (97.5%) identities with a cat sample (BA222) of G3P[9] specificity. The TM H2 samples shared the nt identity of 97.2–99.9% and the aa identity of 98.5–100%. They grouped with P[4] strains into two main sub-clusters (a and b) within lineage III.

The classification of the TM samples by genotypes, lineages, and sublineages on the basis of phylogenetic analysis of the five studied genes is summarized in Table 2. When analyzing the TM samples according to their genotypes, four types of combinations (or genetic constellations) could be observed. The first one consisted of samples that exhibited genotypes G1, G9, or G12 associated with P[8]-I1-E1-H1 and had long electropherotypes. The second one was composed of samples that showed genotypes G2 or G8 associated with P[4]-I2-E2-H2 and had short electropherotypes. The third one included samples exhibiting the G12 genotype combined with I3-E3-H6 and having long electropherotypes, and the fourth one was formed by a sample with genotype G3 associated with P[4]-I2-E3-H3 and having a long electropherotype (Table 2).

Table 2 – Epidemiological data, genotypes, lineages, and sublineages of Triângulo Mineiro rotavirus A strains, based on the VP7, VP8*, VP6, NSP4, and NSP5 genes.

Name of the strains[a]	Patients age	Origin	Month-year of collection	Electropherotype	Genotypes					Lineages and sublineages				
					VP7	VP8*	VP6	NSP4	NSP5	VP7	VP8*	VP6	NSP4	NSP5
Uberaba														
RVA/Human-wt/BRA/UB-09 MG/2005/G1P[8]	1 year	Inpatient (HC)	Sep-2005	Long	G1	P[8]	I1	E1	H1	I-A	3.1	–	–	–
RVA/Human-wt/BRA/UB-16 MG/2005/G9P[8]	2 years	Inpatient (HC)	Sep-2005	Long	G9	P[8]	I1	E1	H1	III	3.1	–	–	–
RVA/Human-wt/BRA/UB-142 MG/2006/GxP[8]	2 years	Outpatient	Jul-2006	Long	Gx	P[8]	I1	E1	H1	–	3.1	–	–	–
RVA/Human-wt/BRA/UB-144 MG/2006/G12Px	1 year	Outpatient	Aug-2006	Long	G12	Px	I3	E3	H6	II	–	–	–	–
RVA/Human-wt/BRA/UB-146 MG/2006/G12P[8]	11 months	Outpatient	Aug-2006	Long	G12	P[8]	I1	E1	H1	III	3.1	–	–	–
RVA/Human-wt/BRA/UB-150 MG/2006/G12Px	4 years	Outpatient	Aug-2006	Long	G12	Px	I3	Ex	H6	II	–	–	–	–
RVA/Human-wt/BRA/UB-157 MG/2006/G9P[8]	1 year	Outpatient	Aug-2006	Long	G9	P[8]	I1	E1	H1	III	3.1	–	–	–
RVA/Human-wt/BRA/UB-169 MG/2006/G9P[8]	1 year	Inpatient (HC)	Aug-2006	Long	G9	P[8]	I1	E1	H1	III	3.6	–	–	–
RVA/Human-wt/BRA/UB-208 MG/2006/G9P[8]	1 year	Outpatient	Sep-2006	Long	G9	P[8]	I1	E1	H1	III	3.1	–	–	–
RVA/Human-wt/BRA/UB-558 MG/2011/G9P[8]	23 months	Inpatient (HC)	Dec-2011	Long	G9	P[8]	I1	E1	H1	III	3.3	–	–	–
RVA/Human-wt/BRA/UB-154 MG/2006/G2P[4]	3 years	Outpatient	Aug-2006	Short	G2	P[4]	I2	E2	H2	IVa-1	IVa	VIIa	VIa	IIIa
RVA/Human-wt/BRA/UB-170 MG/2006/G2P[4]	8 months	Inpatient (HC)	Aug-2006	Short	G2	P[4]	I2	E2	H2	IVa-1	IVa	VIIa	VIa	IIIa
RVA/Human-wt/BRA/UB-226 MG/2006/G2P[4]	5 months	Outpatient	Sep-2006	Short	G2	P[4]	I2	E2	H2	IVa-1	IVa	VIIa	–	IIIa
RVA/Human-wt/BRA/UB-239 MG/2006/G2P[4]	1 year	Outpatient	Oct-2006	Short	G2	P[4]	I2	E2	H2	IVa-1	IVa	VIIa	–	IIIa
RVA/Human-wt/BRA/UB-299 MG/2007/G2P[4]	1 year	Inpatient (HC)	Jun-2007	Short	G2	P[4]	I2	E2	H2	IVa-1	IVa	VIIa	VIa	IIIa
RVA/Human-wt/BRA/UB-320 MG/2007/G2P[4]	11 months	Inpatient (HC)	Aug-2007	Short	G2	P[4]	I2	E2	Hx	IVa-3	IVa	VIIb	VIb	–
RVA/Human-wt/BRA/UB-354 MG/2007/G2P[4]	1 year	Inpatient (HC)	Oct-2007	Short	G2	P[4]	I2	E2	H2	IVa-3	IVa	VIIb	VIb	IIIa
RVA/Human-wt/BRA/UB-432 MG/2008/G2P[4]	1 year	Inpatient (HC)	Sep-2008	Short	G2	P[4]	I2	E2	H2	IVa-3	IVa	VIIb	VIb	IIIa
RVA/Human-wt/BRA/UB-433 MG/2008/G2P[4]	4 years	Outpatient	Sep-2008	Short	G2	P[4]	I2	E2	H2	IVa-3	IVb	VIIb	X	IIIb
Uberlândia														
RVA/Human-wt/BRA/IP-38 MG/2008/G2P[4]	3 years	Inpatient (HSG)	Aug-2008	Short	G2	P[4]	I2	E2	H2	IVa-3	IVb	VIIb	XII	IIIb
RVA/Human-wt/BRA/IP-47 MG/2008/G2P[4]	3 years	Inpatient (HM)	Sep-2008	Short	G2	P[4]	I2	E2	H2	IVa-3	IVb	VIIb	X	IIIb
RVA/Human-wt/BRA/IP-71 MG/2008/G2P[4]	2 years	Inpatient (HSG)	Nov-2008	Short	G2	P[4]	I2	E2	H2	IVa-3	IVa	VIIa	VIb	IIIa
RVA/Human-wt/BRA/IP-346 MG/2010/G2P[4]	1 year	Inpatient (HM)	May-2010	Short	G2	P[4]	I2	E2	H2	IVa-3	IVb	VIIb	VIb	IIIb
RVA/Human-wt/BRA/IP-364 MG/2010/G2P[4]	2 years	Inpatient (HSG)	Jul-2010	Short	G2	P[4]	I2	E2	H2	IVa-1	IVb	VIIb	X	IIIb
RVA/Human-wt/BRA/IP-393 MG/2010/G2P[4]	6 months	Outpatient	Sep-2010	Short	G2	P[4]	I2	E2	H2	IVa-3	IVb	VIIb	X	IIIb
RVA/Human-wt/BRA/IP-218 MG/2009/G3P[4]	4 years	Outpatient	Sep-2009	Long	G3	P[4]	I2	E3	H3	III	IVa	VIIb	–	–
RVA/Human-wt/BRA/IP-447 MG/2011/G8P[4]	5 years	Outpatient	Jan-2011	Short	G8	P[4]	I2	E2	H2	II	IVa	VIIa	X	IIIa

UB, Uberaba; IP, Uberlândia; HC, Hospital da Criança; HSG, Hospital Santa Genoveva; HM, Hospital Madrecor; x, was not possible to amplify.

[a] Name of RV strains according to the nomenclature proposed by Matthijnssens et al.[25]

According to the clustering patterns of the TM G2P[4] samples in the dendrograms for different genes, shown in Fig. 1, some samples always grouped together within the same clusters. However, other samples changed their grouping patterns, clustering with different samples depending on the gene, which is suggestive of intra-genotypic reassortment in particular genes. This was observed for strains IP-364 (VP7 gene), UB-354, UB-432, UB-320, and IP-71 (VP8* gene), IP-71 and IP-393 (VP6 gene), IP-346, UB-239, and UB-226 (NSP4 gene), and UB-354, UB-432, and IP-71 (NSP5 gene). Strains UB-154, UB-170, UB-299, IP-47, UB-433, and IP-38 are probably non-reassortant strains, at least with regard to the genes studied.

Discussion

In the TM samples, genotype G2 was found to be always associated with the P[4], I2, E2, and H2 genotypes, whereas the G1 and G9 genotypes were combined with the P[8]-I1-E1-H1 genotypes (Table 2). The genotype combination G2-P[4]-I2-E2-H2 is characteristic of the DS-1-like genotype constellation, while G1-P[8]-I1-E1-H1 and G9-P[8]-I1-E1-H1 are typical combinations of the Wa-like genotype constellation. These two genotype constellations are found most commonly worldwide.[28]

Despite having a typical DS-1-like constellation for all genes, the TM G2P[4] samples were shown to belong to lineages distinct from that of the DS-1 prototype strain, as already reported for contemporary samples.[16,27,29] Based on the VP7 gene, the G2P[4] samples were detected within sublineage IVa (Fig. 1a). This sublineage emerged in the 1990s and rapidly became the most common sublineage found around the world. The spread of this sublineage was associated with an amino acid substitution from aspartic acid (found in the DS-1 prototype strain) to asparagine at residue 96 (D96N) and with the occurrence of reassortment events in G2 strains.[29] These two mechanisms of variability were also observed in this study (aa data not shown).

Sublineage G2P[4]-IVa-1 of VP7 was found from August 2006 to June 2007 in samples from hospitalized patients and outpatients from Uberaba, followed by the detection of sublineage IVa-3 since August 2007 (Table 2). In Uberlândia, all samples belonged to sublineage IVa-3, except IP-364. Sublineages IVa and IVb of the VP8* gene also seemed to have emerged consecutively in Uberaba. This apparent pattern of temporal segregation of the sublineages of the VP7 and VP8* genes has also been described in other regions of Brazil and for Italian samples.[16,27] An apparent temporal segregation within the G2P[4] lineages from Uberaba was also observed for the VP6, NSP4, and NSP5 genes. Therefore, based on the analyses of the five genes, it seems that a new genetic variant exhibiting the combination of sublineages IVa-3 (VP7), IVb (VP8*), VIIb (VP6), X (NSP4), and IIIb (NSP5) began circulating in Uberaba in September 2008 (UB-433) and was also detected in September 2008 (IP-47) and in September 2010 (IP-393) in Uberlândia, corroborating the results obtained by Gómez et al. who characterized circulation of a new lineage in the southeast region of Brazil.[27]

Samples UB-226 and UB-239 shared high identity in the NSP4 gene with a cow strain (1603). This result is in agreement with the findings of Matthijnssens et al. who revealed a common origin between human DS-1-like and bovine strains.[30]

The G8 genotype is believed to be of bovine origin, but strains with this genotype were also isolated from several other hosts, including humans, and have been detected sporadically in Brazil.[3] The TM G8 sample (IP-447) is closely related to other human RVA strains, such as SS56 from Italy[20] and GER1H-09 from Germany,[31] sharing the same G8-II lineage. There is evidence that strains of this lineage do not represent a result of recent interspecies transmission,[20,31] contrary to what was seen with some G8 strains identified in Brazilian Indian children.[32] The TM G8 sample (IP-447) was genotyped as P[4]-I2-E2-H2, and based on the five genes it was associated with sample GER1H-09, which was described as probably representing an intergenotypic reassortant sample between the G8 genotype and human DS-1-like rotaviruses.[31]

Analysis of the VP7 gene showed that the TM G1P[8] sample (UB-09) detected in 2005, i.e., before the Rotarix™ introduction, belonged to lineage I, which, along with lineage II, represents the most prevalent lineages since the 1990s in Brazil. Through phylogenetic analysis of the VP8* gene, it was possible to verify that UB-09 belonged to lineage P[8]-3, which is believed to be the only lineage currently circulating in Brazil.[33] This sample fell into sublineage P[8]-3.1, which has been shown to be most prevalent in Brazil in recent years.[22]

The phylogenetic analysis of the VP7 gene from the TM G9 strains revealed that they belonged to lineage III and were closely related to strains detected on at least three continents. These results are in agreement with previous findings that showed the worldwide spread of this lineage.[34] When analyzing the VP8* gene, circulation of three sublineages (P[8]-3.6, P[8]-3.3, and P[8]-3.1) was observed in the TM region. Sublineage P[8]-3.6 was only observed in Brazil after the introduction of the Rotarix™ vaccine.[22]

Genotype G12 was first detected in the fecal matter of children from Philippines in 1987. It had not been reported for about 10 years and then rapidly spread globally. It is believed that lineage III was the main lineage responsible for the spread of G12 worldwide.[34] This lineage has been reported to be found most often in combination with the P[6] or P[8] genotypes.[28] One TM G12 sample (UB-146) was found to belong to this lineage and to have the P[8]-I1-E1-H1 genotype combination. This allele combination (Wa-like) is most commonly found for G12, but this genotype can also be found, although at a much lower frequency, associated with P[9]-I3-E3-H3, which are AU-1-like genotypes.[28] Two other TM G12 samples (UB-144 and UB-150) presented AU-1-like genotypes. They also exhibited the H6 genotype, which showed a high nt identity with simian RVA; therefore, it seems that the TM G12 samples have different origins, as has also been observed in another study from Brazil.[35] TM samples UB-144 and UB-150 were closely related to reference strain T152 based on all analyzed genes, and there is evidence that the NSP5 gene of this sample is of animal origin.[19]

According to the phylogenetic trees, the G12-Px-I3-E3/x-H6 strains probably possess P[9] specificity. The number of non-typable RVA strains depends on the number of genotype-specific primers used for RT-PCR genotyping. The genotyping failure is relatively high, given today's technology, and may

represent a low viral load or unusual genotypes (e.g., originating from zoonotic transmission) that are not able to be detected with the primers used.[36] Furthermore, the PCR primers may have become outdated due to mutations in the primer-binding sites during co-circulation of wild-type RVAs over the years.[37]

RVAs of the G3 genotype have several hosts and are commonly found associated with the P[8] genotype in humans. The G3 sample of long electropherotype (IP-218) associated with the P[4] genotype was once detected in the region.[8] Although this combination is unusual, the G3P[4] genotype was responsible for an outbreak of diarrhea in 1998 in Juiz de Fora, Brazil.[38] In the IP-218 sample, a combination with the I2-E3-H3 genotype was found. The evidence of reassortment between G3 samples and typical G2P[4] strains (P[4]-I2-E2-H2) has already been provided[39]; however, to our knowledge, this is the first time when the combination G3-P[4]-I2-E3-H3 is reported.

Based on the VP7 and NSP5 genes, G3 sample IP-218 fell into the cluster of the prototype strain (AU-1) with an RVA strain isolated from cat (BA222). Based on the VP8* and VP6 genes, it was closely related to human rotaviruses that share DS-1-like genotypes. Therefore, the IP-218 sample probably represents a reassortment between AU-1-like and DS-1-like strains, and it would be interesting to characterize the other genome segments of this sample. A close relationship between human G3 RVAs and feline strains has previously been described in Brazil[40,41] and other countries.[42]

The phylogenetic analyses of the five genes from the G2P[4] strains showed that various reassortment events likely occurred from 2006 to 2010 in the TM region, which is consistent with the findings from other studies conducted in Brazil and Italy.[16,27] Both co-circulation of different genetic variants and a significant migration flow between countries contribute to the occurrence of reassortment events. Interestingly, a recent study in Nashville, TN, USA,[43] described the co-circulation of genetic variants that probably only underwent antigenic drifts without the occurrence of reassortments. This disparity in results may be due to a short period (2010–2011) examined in the above study.

In this study, we characterized, based on five genes, the genetic diversity of RVA strains representative of six seasons in the TM region. A great intragenotypic diversity was observed among the G2P[4] samples, with an apparent pattern of temporal segregation within the lineages or sublineages. The occurrence of intergenotypic reassortment events was evident, leading, in particular, to a combination of genotypes not previously reported (G3-P[4]-I2-E3-H3). Five TM samples contained genes that exhibited high nt and aa identities with strains of animal origin. These results highlight the importance of studying diversity of human and animal rotaviruses in order to better understand the mechanisms and the frequency of interspecies transmission. The data of this study cover a period pre- and post-introduction of rotavirus vaccination in all Brazilian territories, thereby serving as a basis for monitoring changes in the genetic constitution of rotaviruses. These changes can influence the viral fitness and the effectiveness of vaccines over time. The present study also contributes to the understanding of the genetic diversity and evolution of rotaviruses in a global context.

Conflicts of interest

The authors declare no conflicts of interest.

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