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Molecular Epidemiology of Contemporary G2P[4] Human Rotaviruses Cocirculating in a Single U.S. Community: Footprints of a Globally Transitioning Genotype

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ABSTRACT

Group A rotaviruses (RVs) remain a leading cause of childhood gastroenteritis worldwide. Although the G/P types of locally circulating RVs can vary from year to year and differ depending upon geographical location, those with G1P[8], G2P[4], G3P[8], G4P[8], G9P[8], and G12P[8] specificities typically dominate. Little is known about the evolution and diversity of G2P[4] RVs and the possible role that widespread vaccine use has had on their increased frequency of detection. To address these issues, we analyzed the 12 G2P[4] RV isolates associated with a rise in RV gastroenteritis cases at Vanderbilt University Medical Center (VUMC) during the 2010-2011 winter season. Full-genome sequencing revealed that the isolates had genotype 2 constellations typical of DS-1-like viruses (G2P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2). Phylogenetic analyses showed that the genome segments of the isolates were comprised of two or three different subgenotype alleles; this enabled recognition of three distinct clades of G2P[4] viruses that caused disease at VUMC in the 2010-2011 season. Although the three clades cocirculated in the same community, there was no evidence of interclade reassortment. Bayesian analysis of 328 VP7 genes of G2 viruses isolated in the last 39 years indicate that existing G2 VP7 gene lineages continue to evolve and that novel lineages, as represented by the VUMC isolates, are constantly being formed. Moreover, G2 lineages are characteristically shaped by lineage turnover events that introduce new globally dominant strains every 7 years, on average. The ongoing evolution of G2 VP7 lineages may give rise to antigenic changes that undermine vaccine effectiveness in the long term.

IMPORTANCE

Little is known about the diversity of cocirculating G2 rotaviruses and how their evolution may undermine the effectiveness of rotavirus vaccines. To expand our understanding of the potential genetic range exhibited by rotaviruses circulating in postvaccine communities, we analyzed part of a collection of rotaviruses recovered from pediatric patients in the United States from 2010 to 2011. Examining the genetic makeup of these viruses revealed they represented three segregated groups that did not exchange genetic material. The distinction between these three groups may be explained by three separate introductions. By comparing a specific gene, namely, VP7, of the recent rotavirus isolates to those from a collection recovered from U.S. children between 1974 and 1991 and other globally circulating rotaviruses, we were able to reconstruct the timing of events that shaped their ancestry. This analysis indicates that G2 rotaviruses are continuously evolving, accumulating changes in their genetic material as they infect new patients.

Group A rotaviruses (RVs), members of the *Reoviridae* family, are known to infect nearly all animal species, including humans, pigs, cattle, and birds, where they are principally associated with diarrheal disease in the young (1). In humans, such infections are the primary cause of life-threatening dehydrating gastroenteritis in children under 5 years of age, resulting in an estimated 453,000 deaths globally in 2008 (2). The RV virion is a triple-layered, nonenveloped icosahedron that contains 11 double-stranded RNA (dsRNA) genome segments (i.e., genes) (3). Together, the viral genome encodes six structural proteins (VP1-VP4 and VP6-VP7) and five or six nonstructural proteins (NSP1-NSP5, sometimes NSP6) (1). In the infected host, neutralizing antibodies are produced against the outer capsid glycoprotein, VP7, and the protease-activated spike protein, VP4 (1, 4). The sequence and antigenic properties of VP7 and VP4 have been used to assign G and P genotypes and serotypes, respectively, to RV strains. In 2008, a full-genome classification system was developed for RVs that also assigns a genotype to each of the internal and nonstructural protein genes (i.e., non-G/P genes) of RVs (5). In this classification system, the acronym Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, where x is an integer, defines the genotype of the VP7-VP4-

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VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 genes of an RV strain (5).

Nucleotide sequencing has revealed the extent of genetic diversity among RVs, with a total of 27 G, 37 P, 18 I, 9 R, 9 C, 8 M, 19 A, 10 N, 12 T, 15 E, and 11 H genotypes recognized by the Rotavirus Working Group to date (6). However, analysis of the >400 complete or near-complete genome sequences of human RVs presently available in the GenBank database indicates that almost all have one of two internal gene constellations (6–10). Represented by 354 available sequences, the Wa-like strains are characterized by genotype 1 non-G/P genes (I1-R1-C1-M1-A1-N1-T1-E1-H1) and G/P genotypes of G1P[8], G3P[8], G4P[8], G9P[8], or G12P[8] (6, 7, 10–12). In contrast, the typical DS-1-like strains, represented by 41 complete sequences, are characterized by genotype 2 internal genes (I2-R2-C2-M2-A2-N2-T2-E2-H2) and tend to have the G/P genotype of G2P[4]. The molecular basis for the existence of the two different internal gene constellations among human RVs is unknown, but it has been suggested that reassortants formed between Wa- and DS-1-like strains are less biologically fit and are at a competitive disadvantage in nature (12, 13). However, recent reports have indicated that in some cases intergenogroup reassortants becomes prevalent, suggesting the ongoing need for whole-genome surveillance (14).

In contrast to Wa-like human RVs, for which studies have examined the genetic diversity and dynamics of both archival and contemporary virus collections, the DS-1-like human RVs have been poorly examined (6, 12, 15, 16). Monitoring of RVs circulating in communities indicates that the G/P genotypes of RVs typically vary from 1 year to the next, with predominant strains like the G2P[4] viruses recycling over a period of 3 to 11 years (15, 17). In recent years, there have been several reports indicating an increase of RV gastroenteritis caused by G2P[4] viruses in communities throughout the world (18–22). Moreover, a lineage of G2 RV strains which carry a hallmark of novel nonsynonymous changes within the antigenic domain of its VP7 protein has become prevalent (23). The accumulation of these VP7 mutations may have increased the fitness of the G2 viruses through neutralization escape, leading to the increased incidence of G2P[4]-associated disease (23).

While global surveillance studies have revealed a recycling pattern of predominant RV G/P genotypes, recent studies have only begun to explore the impact of vaccine introduction on RV diversity and dynamics. The RV vaccines RotaTeq (Merck) and Rotarix (GSK) were licensed for use in the United States beginning in 2006 and 2008, respectively (24, 25). They have led to dramatic decreases in the incidence of severe RV disease and to decreases in RV-associated mortality in some cases (26, 27). RotaTeq is a live-attenuated pentavalent vaccine that contains five different human-bovine reassortant strains. Four of the strains contain a human RV VP7 gene, W179 (G1), SC2 (G2), W178 (G3), and BrB (G4), while one strain confers a human RV VP4 gene, W179 (P[8]) (28). The majority of the internal protein genes of each RotaTeq vaccine component are derived from the bovine RV strain WC3. In contrast, Rotarix is a monovalent vaccine that is comprised of an attenuated human G1P[8] strain (29). While both the monovalent and pentavalent vaccines are effective in preventing severe gastroenteritis caused by RVs with common G/P genotypes (G1P[8], G2P[4], G3P[8], G12P[8]), a debate continues as to their relative effectiveness in inducing heterotypic protective responses (15, 30).

To investigate the extent of RV reassortment and genetic drift in circulating isolates, we sequenced the complete genomes of G2P[4] RVs in 12 stool specimens collected from children with RV gastroenteritis in Nashville, Tennessee, a community with high vaccine coverage. It was estimated that during the 2011 rotavirus season 95% of children under the age of three eligible for vaccination had received at least one dose of either RotaTeq or Rotarix (D. C. Payne, unpublished data). The analysis revealed that all of the isolates contained genotype 2 constellations typical of DS-1-like viruses (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2). Deeper phylogenetic investigation allowed identification of three genetically distinct G2P[4] clades that cocirculated and caused disease during the 2010-2011 season without undergoing reassortment. These findings indicate that the viruses principally evolved by genetic drift and not reassortment. In fact, over the last 39 years, the accumulation of stable point mutations in the VP7 gene has been a frequent event shaping the continuously evolving lineages of G2 viruses, which we predict to arise every 7 years based on Bayesian analysis.

MATERIALS AND METHODS

Sample collection, RNA extractions, and G/P genotyping of RVs. Stool samples have been collected from pediatric patients presenting with acute gastroenteritis at Vanderbilt University Medical Center (VUMC), Nashville, TN, since 1 January 2006 (31, 32). This study was approved by CDC and VUMC institutional review boards. Of the 34 collected samples, 29 deidentified samples were submitted to the NIH for sequencing analysis. The RV-positive stool specimens (n = 29) collected in the 2010-2011 winter season were resuspended to a final concentration of 10% in phosphate-buffered saline. Total RNA was extracted from samples using a QIAamp viral RNA minikit (Qiagen) according to the manufacturer's instructions. The G/P genotypes of RVs in samples were determined by reverse transcription PCR (RT-PCR). Of the 29 samples, 13 were determined to contain G2P[4] RVs (Table 1). Of these 13, one represented a mixed infection that contained both G2P[4] and G3P[8] virus strains; thus, it was not additionally analyzed. To obtain information on older G2 VP7 gene sequences, we analyzed an archival collection of stool samples isolated from pediatric patients hospitalized with gastroenteritis at the Children's National Medical Center (CNMC) in Washington, DC, from 1974 to 1991 (12, 33–37). Total RNA was extracted from 27 samples positive for G2P[4] RV, as determined by microtiter plate hybridization-based PCR enzyme-linked immunosorbent assay (ELISA) (38), using TRIzol (Life Technologies). The 15 samples containing the highest quality RNA were selected for sequencing.

RT-PCR and dideoxynucleotide sequencing. Oligonucleotide primers were designed for each gene based on the consensus of DS-1-like RV sequences available in GenBank. RT-PCR was performed with a SuperScript II one-step RT-PCR kit (Life Technologies). RNA was mixed 1:1 with dimethyl sulfoxide and denatured at 94°C for 10 min prior to cDNA synthesis. RT-PCR products were visualized by electrophoresis and purified using a QIAprep PCR purification kit or a QIAquick gel extraction kit (Qiagen). Amplicons were then sequenced using an ABI Prism BigDye v3.1 terminator cycle sequencing kit and an ABI Prism 3700 genetic analyzer (Applied Biosystems).

Maximum likelihood phylogenetic analyses. Nucleotide sequence contigs were assembled *de novo* using GeneiousPro v6.0.3 (Biomatters). The genotype of each gene segment was determined using RotaC (http://rotac.regatools.be) (39). Nucleotide-level multiple sequence alignments were created from the open reading frame (ORF) of each gene using ClustalW. Amino acid sequence alignments were generated using the BLOSUM-62 matrix of ClustalW. Mega5.05 was used to select the best available substitution model for each alignment according to the Bayesian information criterion (BIC) ranking (40). Maximum likelihood trees were constructed from nucleotide and amino acid

TABLE 1 RV G/P type for samples collected from sick children at VUMC and sequenced by season[a]

G/P type	Season and analysis status[c]											
	2005-2006[b]		2006–2007		2007–2008		2008–2009		2009–2010		2010–2011	
	Coll.	Seq.	Coll.	Seq.	Coll.	Seq.	Coll.	Seq.	Coll.	Seq.	Coll.	Seq.
G1P[8]	64	26	30	10	12	0	4	0	0	0	0	0
G2P[4]	5	0	4	0	0	0	5	0	0	0	12	12
G3P[8]	0	0	2	1	3	0	25	17	1	0	18	0
G9P[8]	0	0	0	0	0	0	2	0	0	0	1	0
G12P[8]	1	0	0	0	0	0	3	2	0	0	1	0
G12P[6]	4	2	0	0	0	0	0	0	0	0	1	0
Mixed	1	0	0	0	0	0	0	0	0	0	1[d]	0
Total	75	28	36	11	15	0	39	19	1	0	34	12

[a] Adapted from McDonald et al. 2012 (13).
[b] For the 2005-2006 season, sample collection began 1 January 2006.
[c] Number of samples collected (Coll.) and sequenced (Seq.) are shown.
[d] Partial sequencing indicates this sample is a mixture of G2P[4] and G3P[8] RVs.

alignments for each gene by applying the selected substitution models with PhyML. Bootstrapping analysis was performed for 1,000 pseudoreplicates. Subgenogroup alleles were assigned to nucleotide trees based on tightly grouped clusters separated by high bootstrap values (>75%). Maximum likelihood trees indicating the relationship of the 12 VUMC RVs to globally circulating strains were constructed using sequences available in GenBank. Accession numbers and sequence alignments will be made available upon request.

Phylodynamic analysis of the VP7 gene. The VP7 gene of the prototypic strain DS-1 (GenBank accession number AB118023) was used as a nucleotide query to gather the 500 closest sequences from NCBI using BLAST (http://blast.ncbi.nlm.nih.gov) (41). Sequences that included the entire ORF and for which the year of isolation was available were selected from this pool and combined with the VP7 gene sequences determined in this study for the 12 VUMC RVs and 15 CNMC RVs. The resulting data set included 328 total VP7 gene sequences (ORFs only) representing G2 RV strains circulating worldwide between 1975 and 2012. These sequences were aligned using ClustalW. The best available model, a general time reversal model with gamma-distributed rate variation (GTR+G), was determined using Mega5.05 (40). Maximum credibility clade (MMC) trees were inferred under a lognormal relaxed clock model and a flexible Bayesian skyline tree prior to using Markov chain Monte Carlo (MCMC) analysis as implemented in BEAST v1.7.3 (42–44). Input files for BEAST were created with BEAUti v.1.7.3. MCMC convergence was obtained with four independent runs of 100 million generations and was evaluated using Tracer (http://tree.bio.ed.ac.uk/software/tracer). Tree and log files were resampled at lower frequency and combined in LogCombiner v.1.7.3. MMC trees were annotated with mean heights using a burn-in of 40,000 as implemented in TreeAnnotator v.1.7.3 (42). All trees were visualized and prepared for publication using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree). The UCSF Chimera molecular modeling system was used to perform structural analysis of the VP7 molecule (PDB number 3FMG; strain RRV) (45, 46).

GenBank accession numbers. The gene sequences of VUMC G2P[4] RVs were submitted to GenBank and assigned the following accession numbers: KF716313 to KF716324 (VP7), KF716325 to KF716336 (VP4), KF716337 to KF716348 (VP6), KF716349 to KF716360 (VP1), KF716361 to KF716372 (VP2), KF716373 to KF716384 (VP3), KF716385 to KF716396 (NSP1), KF716397 to KF716408 (NSP2), KF716409 to KF716420 (NSP3), KF716421 to KF716432 (NSP4), and KF716433 to KF716444 (NSP5). The VP7 genes of archival CNMC G2 RVs were assigned the accession numbers KF716445 to KF716459.

RESULTS

G/P genotypes of RVs in Nashville, Tennessee, during 2006 to 2011. As part of the New Vaccine Surveillance Network, stool samples have been collected at VUMC since 2006 from children under 3 years of age presenting with acute gastroenteritis. As indicated in Table 1, an overall decline in the incidence of severe RV diarrhea occurred in children covered by the surveillance program following introduction of the RotaTeq vaccine in February 2006 and the Rotarix vaccine in April 2008. The most common G/P genotypes of RVs that have been detected at VUMC are representative of G/P genotypes that predominate globally: the G1P[8], G3P[8], and G2P[4] strains. Viruses have also been detected at VUMC, albeit at low frequency, that are representative of globally emerging G/P genotypes: the G9P[8], G12P[8], and G12P[6] strains.

During the initial years of the surveillance program (2006 to 2008), viruses with G1P[8] genotypes were dominant (Table 1). However, in the 2008-2009 winter season, G3P[8] viruses became dominant and few G1P[8] viruses were detected. This season was followed by a year of notably low incidence of severe RV disease, during which only one RV-positive sample (a G3P[8] virus) was collected. Thereafter, in the 2010-2011 season, a diverse group of RVs (12 G2P[4], 18 G3P[8], a single G9P[8], a single G12P[8], a single G12P[6], and a mixture of G2P[4] and G3P[8]) were recovered from children (Table 1). The number of G2P[4]-infected children in the 2010-2011 season was notable, as it represented the greatest number of G2P[4] infections detected in any season in this community since the launch of the surveillance program.

Genotype constellations of VUMC G2P[4] RVs and clade identification. The genotype constellations of the 12 VUMC G2P[4] RVs that infected children during the 2010-2011 season were determined by nucleotide sequencing and analysis of the sequences using the RotaC (version 2.0) website (http://rotac.regatools.be) (39). The results revealed that all 12 had typical DS-1-like genotype 2 constellations (G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2). However, sequences of viral genes and their deduced protein products were not identical (Table 2). Indeed, the VP3 and NSP4 gene sequences of some strains differed by more than 10%, indicating that a single virus strain was not responsible for all of the G2P[4] infections occurring in the 2010-2011 season.

To gain additional insight into the genetic relationships among the G2P[4] viruses, maximum likelihood phylogenetic trees were generated using the ORF nucleotide sequences of the viral genes (Fig. 1). Clusters separated by high bootstrap values and that con-

TABLE 2 Sequence identities of VUMC G2P[4] RVs circulating from 2010 to 2011[d]

Segment	% Cutoff[a]	% nt identity	% aa identity	% nt identity within clade A[b]	% nt identity within clade B[c]	% aa identity within clade A[b]	% aa identity within clade B[c]
VP7	80	96.8–100	98.2–100	100	99.5–100	100	99.4–100
VP4	80	97.3–100	98.5–100	100	99.7–100	100	99.5–100
VP6	85	96.8–100	99.7–100	100	99.8–100	100	100
VP1	83	94.0–100	98.6–100	100	99.8–100	100	99.5–100
VP2	84	97.8–100	99.7–100	100	99.8–100	100	100
VP3	81	87.3–100	93.1–100	100	99.9–100	100	99.8–100
NSP1	79	96.6–99.9	96.2–100	99.9	99.6–99.9	99.8–100	99.2–100
NSP2	85	97.2–100	97.2–100	100	99.5–100	100	99.4–100
NSP3	85	98.4–100	98.7–100	100	99.6–100	100	100
NSP4	85	88.3–100	94.0–100	99.9	99.6–100	100	99.5–100
NSP5	91	98.5–100	99.0–100	100	99.5–100	100	99.5–100

[a] Adapted from Maes et al. (39).
[b] Clade A isolates: VU10-11-2, VU10-11-10, and VU10-11-21.
[c] Clade B isolates: VU10-11-5, VU10-11-6, VU10-11-8, VU10-11-9, VU10-11-11, VU10-11-20, VU10-11-22, and VU10-11-24.
[d] aa, amino acid; nt, nucleotide.

tained unique nucleotide signatures were assigned allele types. Three alleles were defined for all genes except for that encoding NSP5. The three alleles were arbitrarily identified as A (orange), B (purple), and C (green) in Fig. 1. Bootstrap values were not great enough to distinguish the NSP5 allele of the VU10-11-19 isolate from the B-allele cluster; thus, only two alleles (i.e., A and B) were defined for NSP5. The most divergent NSP5 genes of clusters A and B showed only 9 nucleotide differences, pointing to its high degree of sequence conservation.

Analysis of the alleles present in the VUMC G2P[4] RVs allowed us to define three virus clades: A, B, and C (Fig. 2). Clade A viruses were comprised solely of type A alleles for all 11 genes, and clade B viruses were comprised solely of type B alleles. All alleles of the single representative of clade C viruses, VU10-11-19, were

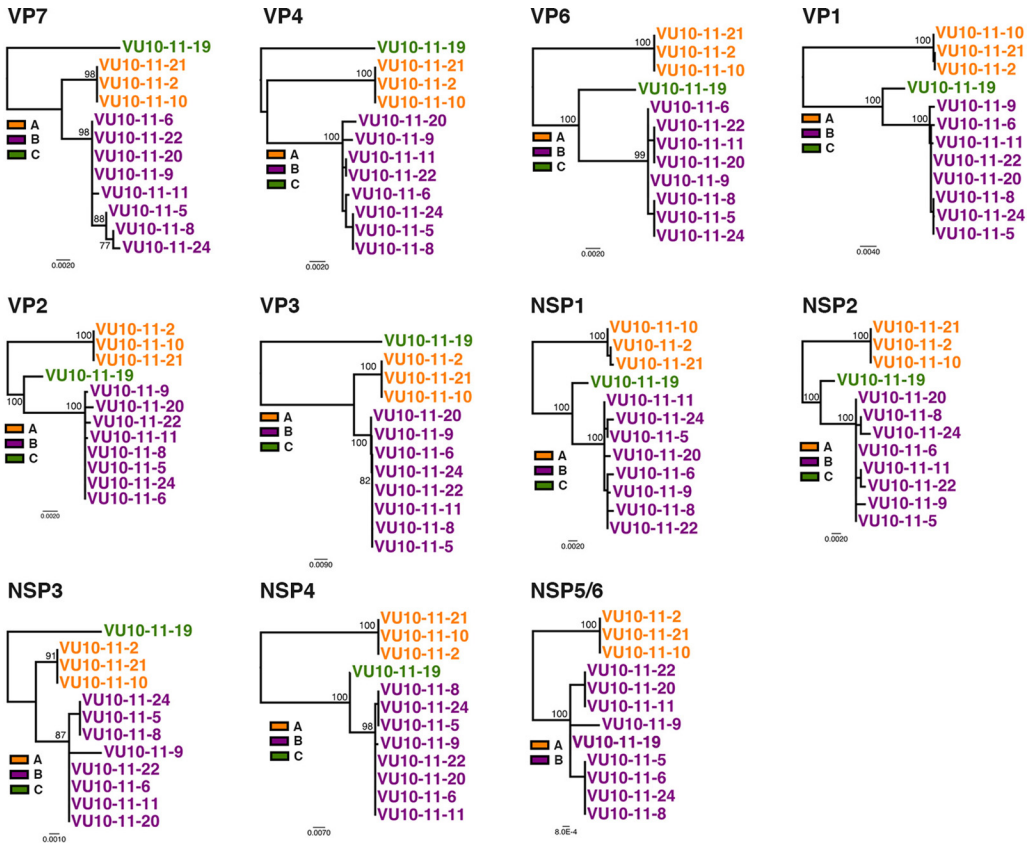

FIG 1 Phylogenetic relationships between each of the 11 genes from cocirculating G2P[4] isolates from VUMC. Maximum likelihood trees were constructed for the open reading frame of each gene and are presented as midrooted. All trees are scaled to the indicated bar. Bootstrap values of ≥75 are reported at each corresponding node by percentage. Color-coded alleles were assigned to clusters separated by high bootstrap values.

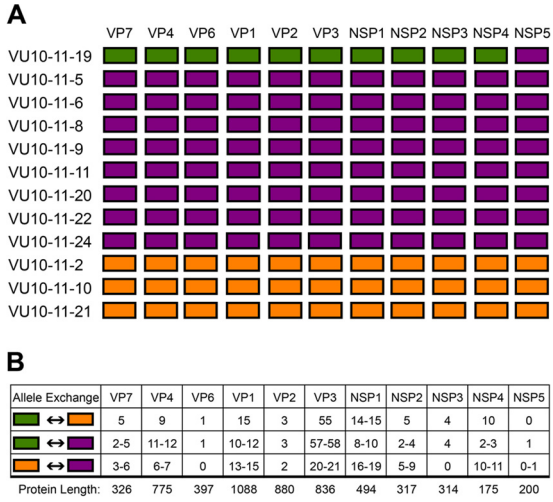

FIG 2 Identification of three major virus clades by allele. (A) Allelic constellations for all 11 genes for each G2P[4] isolate from the VUMC collection as designated in Fig. 1 (A, orange; B, purple; C, green). The isolates are grouped by allelic similarities. (B) Pairwise amino acid differences between allele designations for each gene.

distinct from those of clade A viruses and, perhaps with the exception of the NSP5 gene, from those of clade B viruses. Viruses belonging to the same clade contained alleles with nucleotide identity values that were very high, in many cases reaching 100% (Table 2). The uniformity of allele types of the G2P[4] viruses belonging to the same clade indicates that reassortment did not occur between viruses of the three clades despite their cocirculation in the Nashville area during the 2010-2011 season.

Notably, the relative genetic distance between the alleles of clade A, B, and C viruses varied from gene to gene. For example, allele C of the VP3 gene was more distantly related to alleles A and B than A and B were to each other. In contrast, allele A of the VP1 gene was more distantly related to alleles B and C than the latter two were to each other. These gene-to-gene differences suggest that the evolution of the G2P[4] clades was shaped by reassortment between progenitor virus strains before their introduction into the Nashville area.

VUMC G2P[4] clades encode divergent proteins. Amino acid sequence alignments were constructed to identify differences in proteins encoded by viruses belonging to the 3 VUMC G2P[4] clades (data not shown). The results showed that most, if not all, of the 11 viral proteins of one clade differed in sequence from the proteins of another clade. The number of amino acid differences ranged from none to relatively few for most of the viral proteins but tended to be higher for VP1, VP3, and NSP1 (Fig. 2). Although the VP3 sequences of the clade B and C viruses differed by 57 to 58 residues, these were not concentrated in any particular region of the protein.

The amino acid compositions of the VP7 7-1a, 7-1b, and 7-2 antigenic domains of the clade A, B, and C viruses are nearly the same (Fig. 3) (12). Perhaps the most notable is the surface-exposed D120N change noted in the 7-1A domain of two clade B isolates. There are only two previous reports of a G2 virus with a D120N-substituted VP7 protein. These viruses were isolated in Brazil in 2005 and Italy in 2011, and both contain divergent resi-

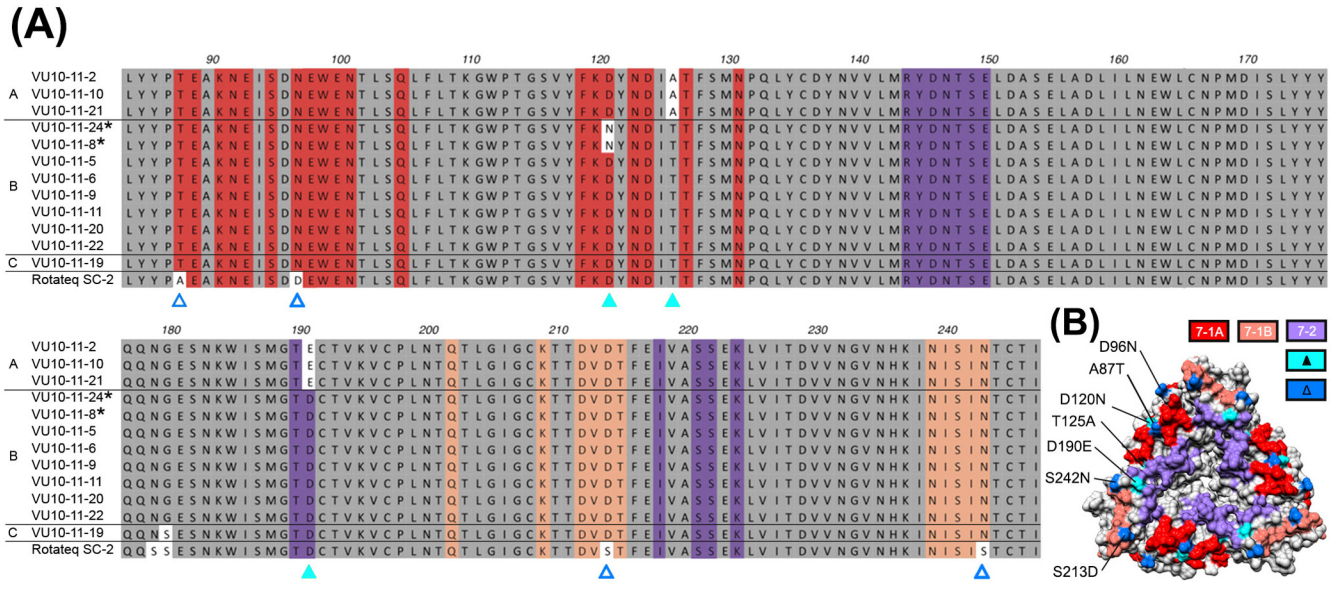

FIG 3 Comparison of the antigenic residues encoded by the VP7 genes of the VUMC G2 isolates to SC2-9, the G2 component of RotaTeq. (A) Alignment of residues 65 to 246 of the VP7 gene comparing the three VUMC clades, A, B, and C, to the SC-2 component of RotaTeq. Columns are colored based on the antigenic domain the residues lie in: red, 7-1A; salmon, 7-1b; and purple, 7-2. Amino acids that differ from the consensus are in white. If this change has been routinely observed in circulation, their positions are marked with a blue outlined triangle. If this change has not been routinely observed in circulation, their positions are marked with a filled-in cyan triangle. Filled-in triangles denote amino acid changes not previously reported. Outlined triangles denote amino acid changes that appear to be stable in modern G2 RVs. (B) The trimer reconstruction of the G3 VP7 protein from RRV seen from the top (PDB code 3FMG). The antigenic epitopes are colored red (7-1A), salmon (7-1b), and purple (7-2). Observed amino acid changes between RotaTeq and the VUMC isolates commonly seen in circulation are indicated in blue and are labeled according to mutation. Observed amino acid changes between RotaTeq and the VUMC isolates not commonly seen in circulation are indicated in cyan and labeled according to mutation.

dues at other sites (10, 47). The VUMC clade A viruses also contain residues that differ from the clade B and C viruses; one change (E190D) is located within the VP7 7-2 antigenic domain and has not been detected previously among US G2 virus strains. The clade A viruses also contain an unusual T125A change. However, because the residue is somewhat masked by other residues of the 7-1 domain, its significance to antigenicity is not clear. Comparison of the VUMC G2P[4] viruses to the G2 virus component of RotaTeq revealed 4 amino acids in the antigenic domain. Two were in the 7-1A domain (A87T and D96N) and two in the 7-1B domain (S213D and S242N). This combination of changes has been described as a stable feature of modern G2 isolates (23).

Comparisons of the VP4 segments of the VUMC G2P[4] viruses to those of other P[4] viruses deposited in GenBank identified several residues that were unique to the VUMC collection. However, only one was within the predicted antigenic domain of the VP8* and VP5* proteolytically processed fragment of VP4. Notably, the VP4 protein of isolate VU10-11-9, a clade B virus, contains a unique amino acid change, N161T, which is located in the 8-3 domain of VP8* and has not previously been reported for a P[4] RV.

G2P[4] VUMC RVs are closely related to globally circulating human and animal DS-1-like strains. To identify the evolutionary relationships and possible reassortment events that shaped the G2P[4] viral clades circulating in Nashville, the ORF sequences of each gene allele were matched with similar sequences available in GenBank. The results show that isolates with genes similar to those of the G2P[4] VUMC RVs were found circulating in the last decade, including isolates RVA/Human-wt/BGD/MMC6/2005/G2P[4], RVA/Human-wt/USA/LB2744/2006/G2P[4], RVA/Human-wt/USA/LB2772/2006/G2P[4], RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/BGD/RV161/2000/G12P[6], and RVA/Human-wt/DEU/GER1H-09/2009/G8P[4] (Fig. 4) (48–52). Most of the genes from the 12 VUMC viruses clustered most closely with those of other G2P[4] strains, but several internal genes from the 12 VUMC viruses clustered with genotype 2 genes from isolates with different G/P genotypes. For example, 1473 is a G8P[4] strain and RV161 is a G12P[6] strain; the genes of both of these strains cluster tightly with those of the VUMC RVs and are known to contain genotype 2 backbones. Overall, the G2P[4] VUMC RVs formed the same clustering pattern in lineage trees populated with all of the closely related strains as those from the cocirculating isolates alone (Fig. 1 and 4). The addition of closely related global strains emphasizes that the relationship between the three clades is polyphyletic and that the three clades represent individual evolutionary virus entities (Fig. 4).

Among the G2P[4] VUMC RVs, proteins VP3 and NSP4 appeared to be the most divergent given the length of these segments, encoding 58 different residues out of 836 and 10 out of 175, respectively (Fig. 2B). This divergence is reflected in the phylogenetic lineages derived from their nucleotide sequences (Fig. 4). In particular, for the NSP4 gene, isolates appear to split into two branches, human RV-like and bovine RV-like strains. Genes of clade A RVs cluster with strain RVA/Human-wt/JPN/KO-2/2004/G2P[4] (AF401756), which was isolated in Japan from an elderly patient during an outbreak in 2004 (Fig. 4) (53). Genes of clade B and C RVs fall on a branch heavily populated by RVs of bovine origins, for example, RVA/Bovine-wt/IND/RUBV81/G10P[14], which came from a collection isolated between 2001 and 2005 in India (54). In the case of the VP3 gene, VU10-11-19 groups closely with RVA/Human-wt/BGD/MMC88/2005/G2P[4] (HQ641366). This gene of both strains shares a common ancestor with RVA/Goat-tc/BGD/GO34/1999/G6P[1] (GU937879), a strain isolated from a goat in Bangladesh in 1999, and several other human strains, including RVA/Human-wt/HUN/BP1062/2004/G8P[14] (FN665690) and RVA/Human-wt/EGY/EGY3399/2004/G6P[14] (HM113527), which are believed to be of animal origin (55, 56). Although separated by time, 1999 and 2005, respectively, GO34 and MMC88 were both collected in the same area of Bangladesh (48, 57). The strain GO34 also appears to have shared a closely related VP1 gene (GU937877) with the G2P[4] VUMC RVs of clades B and C. The diversity exhibited by the VP1, VP3, and NSP4 genes of clade B and C RVs indicates an ancestral relationship to animal RV strains.

The G2 VP7 gene of VUMC RVs belongs to a rapidly emerging lineage. If the introduction of RV vaccines places circulating strains under additional selective pressure, a high rate of mutation could allow them to adapt more quickly. To examine the global evolutionary dynamics that shaped the emergent G2P[4] VUMC RVs, we used BEAST, a software package that can construct time-measured phylogenies. The phylogeny was constructed from the ORF sequences of 328 G2 RV VP7 genes isolated globally between 1975 and 2012. The mutation rate of the G2 VP7 gene was estimated to be 1.45×10^{-3} mutations per site per year with a 95% confidence interval of 1.12×10^{-3} to 1.63×10^{-3} (Fig. 5). The coefficient of variation, 0.84, indicates that the rate varied over 84% of the tree, and the 95% confidence interval, 0.63 to 1.05, clearly excludes zero, suggesting departure from a strict molecular clock model (58). Together these values indicate that there is a variation in rates among the majority of the tree branches; this informed our choice of a relaxed-clock model, which allows the rate to vary from branch to branch (59).

The phylogenetic history of the G2 VP7 gene illustrates the rise of four major lineages, I to IV, and three sublineages, IVa-1 to IVa-3, as defined by Doan et al. in 2011 (23). The most recent common ancestor of the VP7 gene seen in all globally circulating human G2 RVs is predicted to have existed in 1949 (1927 to 1965), decades before the collection of the prototypic DS-1 strain in 1976 (Fig. 5). Some descendants of this ancestor gene split into lineage II, which contains descendants of strains isolated in Australia and Africa from 1992 to 1998. In 1956 (1941 to 1965), lineage I arose and was representative of the prototypic strain DS-1. Lineage III appears to have split off in 1968 (1957 to 1975). This phylogenetic model dates the emergence of lineage IV to around 1976 (1971 to 1979). Lineage IV continued to expand, diverging into sublineage IVa-2 in 1984 (1981 to 1985), sublineage IVa-1 in 1986 (1983 to 1988), and sublineage IVa-3 in 1989 (1985 to 1991). By calculating the difference between the most recent common ancestor dates between the G2 RVs (1949), lineage I (1956), lineage III (1968), lineage IV (1976), sublineage IVa-2 (1984), sublineage IVa-1 (1986), and sublineage IVa-3 (1989), we calculated the average time between divergence events to be 7 years.

Despite their widespread circulation, certain VP7 gene lineages appear to have died out over time (Fig. 5). Close evaluation of the 15 archival CNMC G2 RVs, which are lineage I representatives, reveals the timing and finite period of their circulation (Fig. 6). The VP7 genes from the two oldest RVs from 1975 to 1976 represent one clade, those from RVs circulating in 1977 to 1980 are divided between clades, and those from RVs isolated in 1991 form a fourth. To our knowledge, isolates DC54444, DC5520, DC5357,

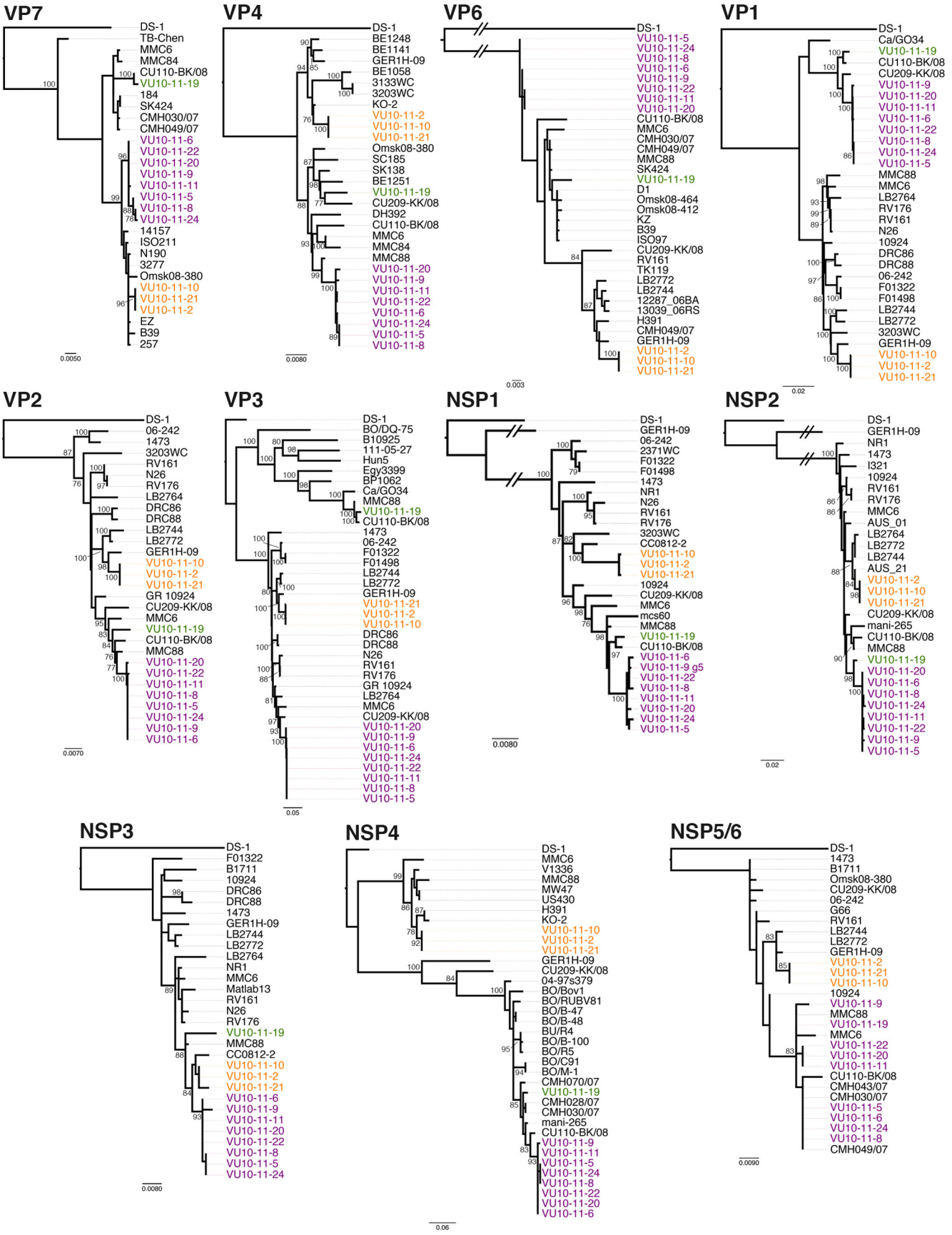

FIG 4 Phylogenetic relationships between each of the 11 G2P[4] genes in circulation in Nashville, TN, and close relatives circulating globally. Close relatives of each previously designated allele were determined from a BLAST query of a representative isolate. Maximum likelihood trees were constructed for the open reading frame of each gene. The prototypic strain DS-1 was used to root all trees, which are scaled to the indicated bar. Bootstrap values of ≥75 are reported at each corresponding node by percentage. Color-coded alleles (A, orange; B, purple; C, green) were used as described for Fig. 1.

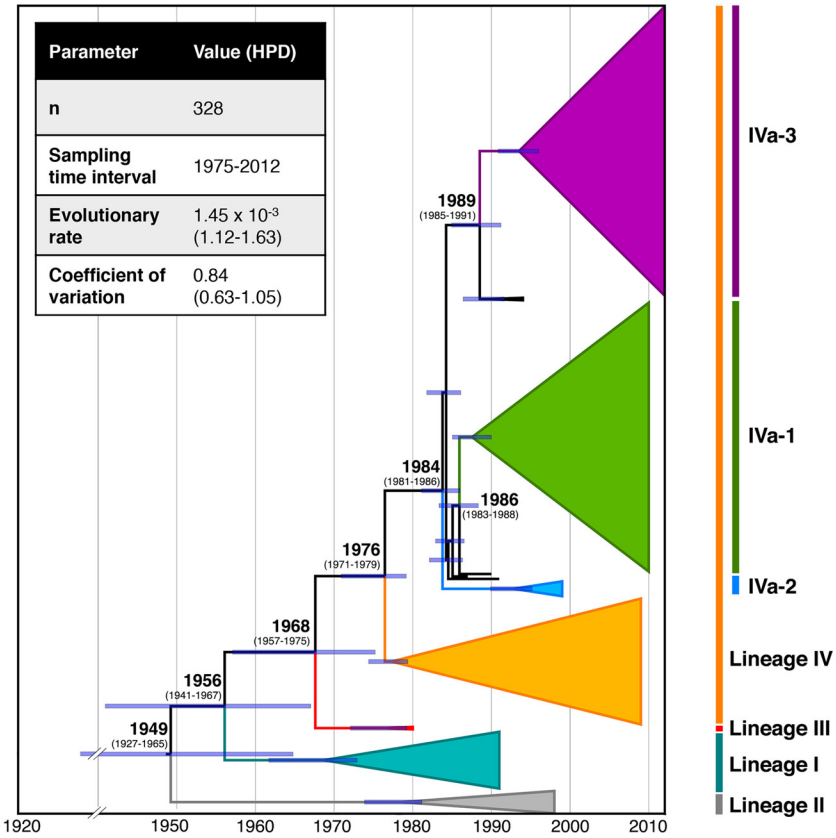

FIG 5 Simplified maximum clade credibility tree of 328 dated G2 VP7 ORF nucleotide sequences. The 95% highest posterior density (HPD) interval of each significant node is indicated with a blue bar. The time of the most recent common ancestor is indicated for each lineage. Major lineages have been collapsed for simplicity (lineage I, teal; lineage II, gray; lineage III, red; lineage IV, yellow; sublineage IVa1, green; sublineage IVa2, blue; sublineage Iva3, purple). The height of each triangle represents the number of sequences in the lineage, while the width represents the genetic diversity. The inlaid table presents parameters of the G2 data set.

and DC5806, collected in 1991, mark the last reported observance of G2 RVs with lineage I VP7 genes. The sequence identity of archival CNMC VP7 is markedly lower than those of modern strains. The VP7 genes of these CNMC RVs shared 98.2 to 98.8% nucleotide identity with the prototypic DS-1 strain, contrasting with those of the recent VUMC RVs, which show 92.8 to 93.3% identity to the prototypic DS-1 strain (data not shown).

RVs with lineage IV VP7 genes remain an epidemiologically important cause of disease and have replaced viruses with lineage I, II, and III VP7 genes. The VP7 genes of the G2P[4] VUMC isolates all cluster within sublineage IVa-3, which emerged around 1989 (1985 to 1991) (Fig. 7). Their most recent common ancestor likely circulated in 1999 (1997 to 2001). Clade B VP7 genes separated in 2006 (2005 to 2007). The VP7 gene of clade C appears to have been circulating since 2008. The single clade C isolate, VU10-11-19, clusters closely with a strain from Thailand that was collected in 2008, RVA/Human-wt/THA/CU110-BK/2008/G2P[4] (GQ996882). The VP7 gene of clade A RVs emerged in 2009 (2008 to 2010). Clade A and B VP7 genes are most closely related to VP7 sequences collected in Russia between 2008 and 2012, including RVA/Human-wt/RUS/Nov-09EZ/2009/G2P[4] (GU377166), and appear closely derived from RVA/Human-wt/USA/LB2764/2006/G2P[4] (HM467955), a strain isolated in Long Beach, California, in 2005. The clade B viruses, VU10-11-8 and VU10-11-24, which contain the substitution D120N in unique combination with A87T, D96N, S213D, and S242N, appear to have emerged some time after the 2009-2010 RV winter season. The time-calibrated phylogeny indicates they share an ancestor with Vanderbilt isolate VU10-11-5, which, like the other VUMC isolates, does not contain the D120N mutation. These results suggest ongoing evolution of the G2 IVa-3 lineage in the Nashville, Tennessee, area.

DISCUSSION

Virus surveillance programs at VUMC have provided an opportunity to monitor the diversity and evolution of RV strains circulating in a postvaccine community. Overall, a decrease in severe RV disease has been observed in Nashville, Tennessee. The 2010-2011 season marked the steepest season-to-season increase in observed cases since 2005. Complete genome sequencing of the 12 G2P[4] VUMC RVs, which contributed to 12 of 34 infections observed during the winter of 2010 to 2011, revealed that they all exhibited DS-1-like genotype constellations. These findings are consistent with previous results that suggest reassortants between DS-1-like and Wa-like viruses are rare (15).

The G2P[4] VUMC RVs appear to have originated from the separate introduction of three ancestral viral strains to the Nashville area and are similar to those in global circulation. Phylogenetic analysis of 10 genes of the 2010-2011 G2P[4] VUMC RVs, those encoding VP1-VP4, VP6, VP7, and NSP1-NSP4, revealed that they consistently formed three distinct clusters (Fig. 2A).

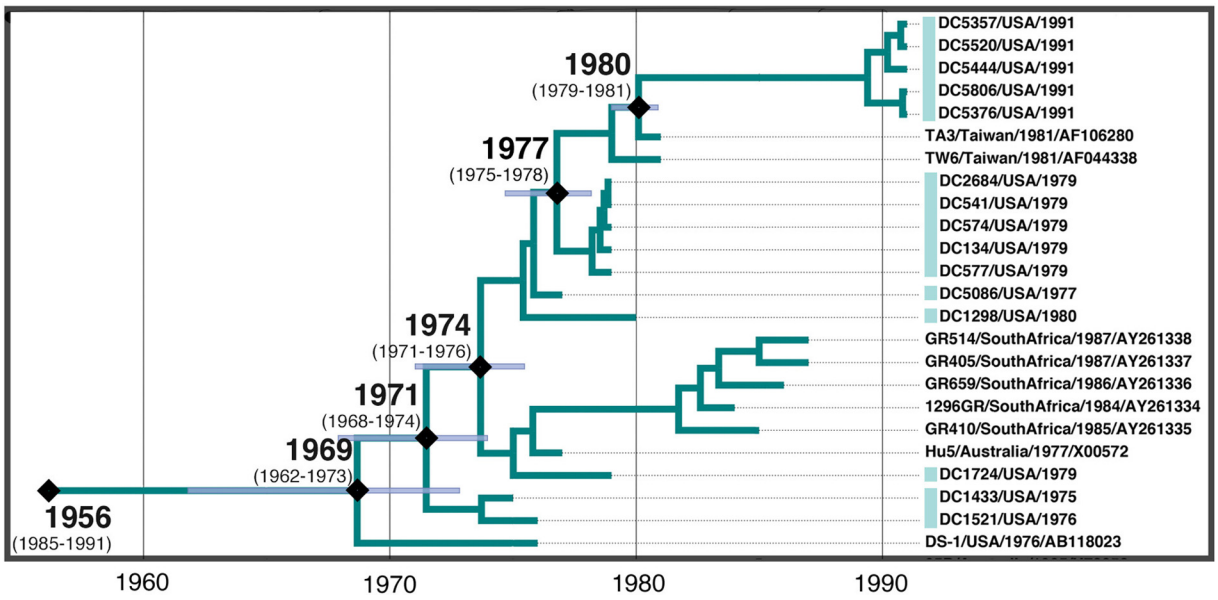

FIG 6 Detailed view of lineage I from Fig. 5. The 95% HPD interval of each significant node (black diamond) is indicated with a blue bar. Times of the most recent common ancestor of these lineages are indicated. CNMC isolates are indicated with a teal rectangle.

Plotting the allelic designations of each cluster for each gene revealed 3 G2P[4] viral clades which did not undergo reassortment (Fig. 2A).

Distinct clades of Wa-like isolates have been observed cocirculating in Nashville and other communities (12, 13, 37), and distinct clades among DS-1-like viruses have been described here. The genetic lineages of the cocirculating VUMC RVs suggest they do not form a single monophyletic group and are most likely of polyphyletic origins, in that each of the three clades appears to be recently shaped by different evolutionary histories (Fig. 4). Gene sequences from the three clades share a high level of similarity with those of strains collected from the United States, Asia, Europe, and Africa from 2001 to 2011, although these relationships were not consistent between trees of different gene sequences. Reassortment is facilitated by a high rate of coinfection, which is typically much higher in developing countries (15, 60). If the three clades were transplanted to Nashville, preshaped by the unique environmental pressures characteristic of developing countries, they may not have experienced sufficient opportunity or pressure to reassort further, resulting in pure allele constellations. Nevertheless, they do appear to have undergone genetic drift since their introduction.

The VP1, VP3, and NSP4 genes of certain clades had the greatest level of amino acid divergence, as high as 6.8% between isolates (Table 2), and appear to share a distant common ancestor with those of animal strains. Reassortment between animal and human strains is predicted to rapidly alter RV diversity by introducing novel gene constellations (12, 61, 62). The relative similarity between the clade B and C NSP4 alleles and those of the modern bovine strains supports the existence of a distant common ancestor between human- and bovine-derived RVs (Fig. 4). Similarly, the caprine RV-like VP1 of the clade B and C viruses and the caprine RV-like VP3 gene of the clade C virus were similar to those of strains isolated from ruminants in developing areas of Bangladesh and India. Similar constellations incorporating one and sometimes two of these genes have recently been observed in stable circulation among children in Italy (63). Animal strains may need to undergo advantageous evolution and further reassortment before being adapted for efficient replication and transmission in humans (64). Clade C, represented by a single isolate, VU10-11-19, did not appear to be as widespread as clades A and B, represented by three and eight isolates, respectively (Fig. 2B). While each of the potential animal VP1, VP3, and NSP4 alleles have been observed in circulating strains, the analysis of the VU10-11-19 isolate is the first description of a unique constellation incorporating all three of these genes (Fig. 4). Despite its capacity to infect a human child, VU10-11-19 may represent a strain not yet adapted for efficient human-to-human transmission. Due to the limited surveillance of typical caprine and bovine RVs, it remains unclear whether the observed increase of these potential animal alleles is due to the frequent spillover of genes from animal RVs or a reflection of the gradual adaptation of stable constellations suited to efficient spread and replication in humans.

The evolving G2 strains appear to quickly overcome geographic barriers, yet some lineages are more persistent than others over time. The gradual acquisition of point mutations in the VP7 gene has facilitated the expansion of G2 diversity during the last century. The mutation rate calculated for the G2 VP7 gene (1.45×10^{-3}) is similar to that of the G9 RVs (1.87×10^{-3}) and G12 RVs (1.66×10^{-3}), which spread globally in less than a decade (65). This large-scale phylodynamic analysis reveals the chronological emergence and evolutionary dynamics of globally circulating G2 RVs (Fig. 5). Our model indicates that RVs with modern G2 genotypes emerged during the late 1940s. Since then, the G2 RVs in circulation today have undergone several events of expansion and lineage replacement. The original major lineages, I, II, and III, seem to have lost epidemiological importance by 2000. For example, all of the G2 CNMC RVs, which were collected between 1975 and 1991, have VP7 genes that closely resemble those of the archi-

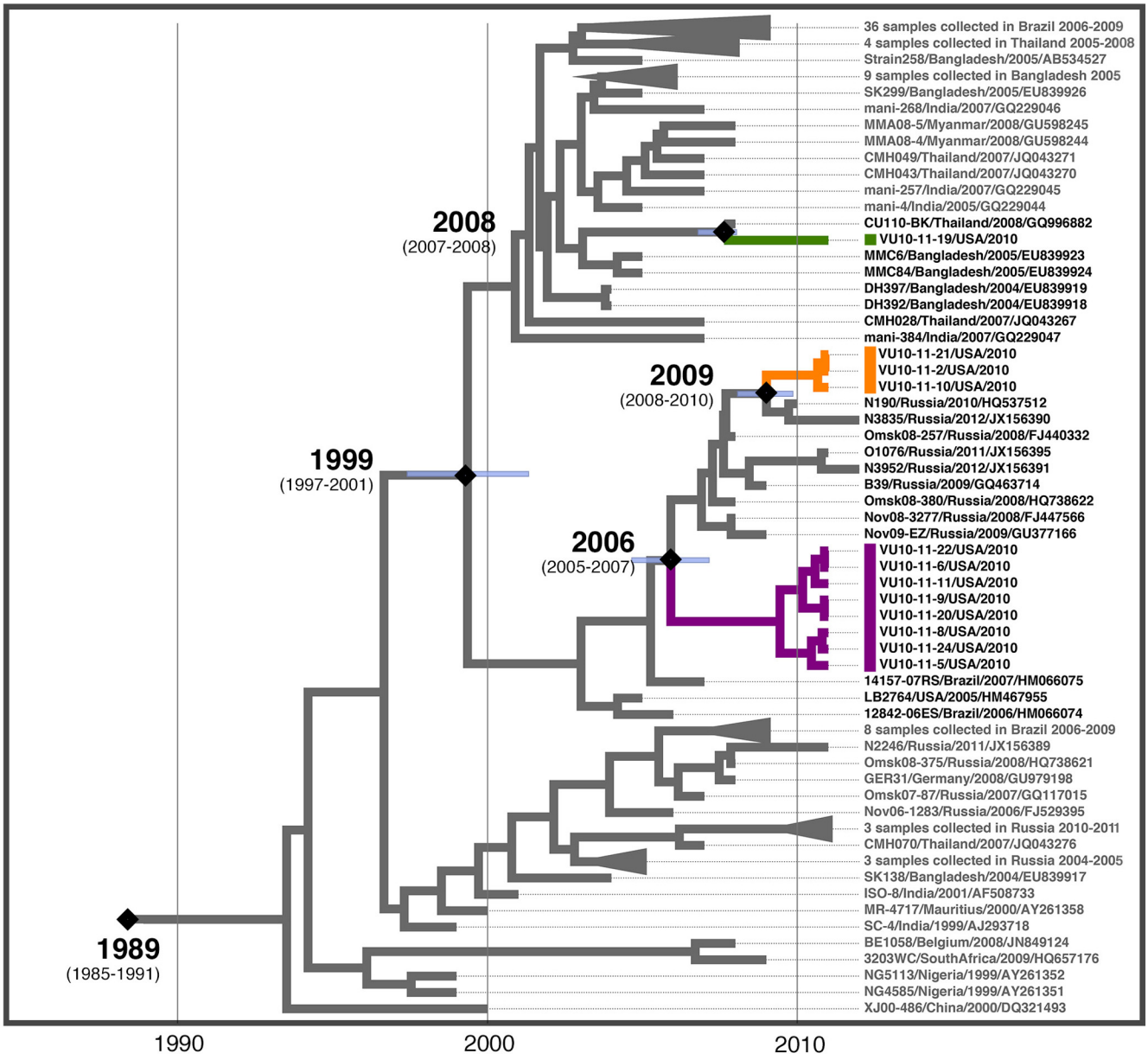

FIG 7 Detailed view of sublineage IVa3 from Fig. 5. The 95% HPD interval of each significant node (black diamond) is indicated with a blue bar. Times of the most recent common ancestor of these lineages are indicated. The clade A (orange), clade B (purple), and clade C (green) alleles are colored as designated in Fig. 1. Close phylogenetic relatives of the VUMC isolates are indicated in boldface. More distantly circulating IVa3 viruses are labeled in gray.

val strains of lineage I (Fig. 6). To our knowledge, these isolates are the last reported lineage I G2 viruses. Even among the CNMC strains, those collected from 1991 had VP7 genes that formed a clade distinct from those of RVs collected in 1975 to 1976, yet none were closely related to modern strains. Lineage IV, which emerged around 1976 (1971 to 1979), has evolved as a significant and persistent contributor to disease, representing 100% of G2 VP7 genes from modern isolates (23). The incidence of RVs with the G2P[4] serotype has been shown to oscillate (15, 17). From our global phylodynamic analysis, it appears that periodic revivals of G2P[4] RVs are the result of new VP7 gene lineages (Fig. 5). The average time between lineage divergence events in the G2 phylogenetic history is calculated to be 7 years. The persistent ability of the G2 viruses to evolve through cyclical lineage turnover underlines their potential durability despite short-term fluctuations.

Observations of epidemiological data suggest that G2P[4] RVs are particularly adept at causing disease in elderly adults (66–68). One possible explanation is their reduced immunity to DS-1-like viruses, which have undergone extensive evolution since their childhood and circulated at low frequencies throughout their lifetimes. In the last 20 years, the lineage IV G2 VP7 genes have completely replaced those of all other lineages and may pose a unique antigenic challenge.

The branching of G2P[4] VUMC RV clades A and B indicates that these viruses are continuing to evolve in a postvaccine world (Fig. 7). Isolates from each of these clades expressed unique com-

binations of amino acid residues in the antigenic domain of their VP7 protein and were different from vaccine strains (Fig. 3). Mutations in antigenic epitopes have been reported in other vaccinated communities, but it is not apparent that these small changes have undermined the effectiveness of the RotaTeq and Rotarix vaccines, which may provide protection through a heterotypic response (15). Rather, these changes are regarded as indicators that these strains can antigenically adapt. Antigenic differences, together with a relatively high mutation rate and the possibility of genetic reassortment, may compound the capacity of G2P[4] viruses to cause infections in the young and the elderly, resulting in regional spikes in RV infections. The success of these viruses appears to be limited by an unexplained preference for particular gene constellations, perhaps on the allelic level. However, viruses that have already undergone complex gene evolution and reassortment, adapting them to efficient replication in humans, may rapidly spread once introduced to a community, resulting in lineage turnover. Further, full-genome surveillance of season-to-season isolates is necessary to refine our understanding of the genetic diversity, evolutionary dynamics, and regional relationships of circulating viruses in this postvaccine world.

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