

Detection of G3P[3] and G3P[9] Rotavirus Strains in American Indian Children With Evidence of Gene Reassortment Between Human and Animal Rotaviruses

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The distribution and evolution of human rotavirus strains is important for vaccine development and effectiveness. In settings where rotavirus vaccine coverage is high, vaccine pressure could select for replacement of common strains (similar to those included in rotavirus vaccines) with uncommon strains, some of which could be generated by reassortment between human and animal rotaviruses. Between 2002 and 2004, a phase-III rotavirus vaccine clinical trial was conducted among American Indian children of the Navajo and White Mountain Apache tribes, which are known to be at high risk for rotavirus diarrhea. We evaluated the rotavirus strains collected from study participants who received placebo during the trial to determine the distribution of rotavirus genotypes and to detect emerging strains that contribute to disease and could influence rotavirus vaccine effectiveness. Three uncommon strains of human rotavirus, two G3P[3] and one G3P[9] strains were detected in stools of children aged 3 to 6 months of age. Segments of all 11 rotavirus genes were sequenced and genotyped by comparison of cognate gene fragments with reference strains. The G3P[3] strains had similar genotypes to each other and to reference dog and cat strains. The G3P[9] strain had similar genotypes to cow, cat and dog reference strains. Genetic analyses of these three strains support the known diversity generating mechanisms of rotavirus. **J. Med. Virol.** 83:1288–1299, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: G3P[3] genotype; G3P[9] genotype; cat rotavirus; dog rotavirus; rotavirus vaccine; American Indian

INTRODUCTION

Diarrhea is among the two most common causes of death among children less than five years of age in the developing world and is responsible for approximately 1.5 million deaths each year [United Nations Children's Fund, 2007]. Rotavirus is the most frequent etiologic agent of severe diarrhea among children resulting in an estimated 600,000 deaths per year [Parashar et al., 2006]. In the United States (U.S.), an estimated 60,000 rotavirus diarrhea hospitalizations occur annually among children resulting in \$246 million of medical costs and \$1 billion in costs overall [Tucker et al., 1998; Fischer et al., 2007]. Various factors have been associated with increased risk of severe rotavirus disease or hospitalization in the U.S. including low birth weight, young maternal age, failure to breastfeed and attending day-care centers [Parashar et al., 1998; Dennehy et al., 2006; Newman et al., 1999]. Additionally, previous studies have shown that some American Indian populations have a high risk of severe diarrhea [Santosham et al., 1985; Santosham et al., 1995].

Rotaviruses belong to the family *Reoviridae*, which are non-enveloped, icosahedral viruses with a double-stranded RNA (dsRNA) genome of 11 segments. Three concentric layers of proteins enclose the dsRNA. The outer capsid layer is composed of two proteins, VP4

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and VP7, which play an important role in virus entry and infection of the target cell. Both VP4 and VP7 have been targeted by vaccine developers because of their role in inducing a neutralizing antibody response during infection [Estes and Kapikian, 2007]. Two rotavirus vaccines have been licensed in the U.S. and in clinical trials prevented more than 85% of severe rotavirus disease. Both vaccines have demonstrated cross protection against rotavirus types that are not contained in the vaccine, but this degree of protection is variable [Ruiz-Palacios et al., 2006; Vesikari et al., 2006]. Increasing diversity in circulating rotavirus genotypes could result in emergence of non-vaccine types that could escape immunity elicited by currently available vaccine formulations.

There are seven rotavirus serogroups, A-G, as defined by antigenic determinants found on structural proteins, such as the intermediate capsid protein, VP6, and likely to be found on nonstructural proteins. Groups A-C infect both humans and animals, while groups D-F have only been detected in animals [Estes and Kapikian, 2007]. Group A rotavirus is the most common cause of gastroenteritis, especially among children. Group A strains can be further classified into one of four subgroups (subgroup I, subgroup II, both subgroup I and II or no subgroup) based on reactivity patterns with subgroup-specific monoclonal antibodies (MAbs) directed to VP6 [Estes and Kapikian, 2007].

A binary classification scheme has historically been used to categorize rotavirus strains by serotype or genotype. Serotype is determined by serum neutralizing antibody results while genotype is determined by PCR and sequence analysis. The binary classification scheme assigns serotype and genotype according to the G type (glycoprotein) and P type (protease-sensitive) as encoded by VP7 and VP4 genes, respectively. Rotavirus classification methods have evolved primarily from antibody-based assays (i.e. serotyping) to genetic characterization (i.e. genotyping) because sequence analysis is a relatively straightforward process.

Recently, a new rotavirus classification scheme based on nucleotide sequencing of all genome segments was proposed by Matthijnssens et al. [2008a] and a rotavirus classification working group (RCWG) [Matthijnssens et al., 2008b] and endorsed by the International Committee of taxonomy (ICTV). The new classification system assigns a genotype to each of the 11 rotavirus proteins as follows: Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx for VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, respectively. Currently, as many as 23 G genotypes and 31 P genotypes have been detected in both mammalian and avian populations [Matthijnssens et al., 2008a; Schumann et al., 2009; Ursu et al., 2009]. According to this classification system, 13 I (Intermediate capsid shell, VP6), 6 R (RNA polymerase, VP1), 6 C (Core shell, VP2), 7 M (Methyltransferase, VP3), 16 A (interferon Antagonist, NSP1), 6 N (NTPase, NSP2), 8 T

(Translation enhancer, NSP3), 12 E (Enterotoxin, NSP4), and 8 H (Phosphoprotein, NSP5) genotypes have been established and published [Matthijnssens et al., 2009a].

Historically, there have been four predominating Group A rotavirus strains that constituted at least 88% of strains detected globally among children with diarrhea: G1P1A[8] (52–65%), G3P1A[8] (3%), G4P1A[8] (8.5%) and G2P1B[4] (12%) [Gentsch et al., 2005; Santos and Hoshino, 2005]. Recently identified strains that contain antigens G5, G8, G9, G12 and P[6] have emerged as regionally or globally important strains. Of the most prevalent global strains, G3 strains account for 12% of typed rotavirus strains and have been detected in combination with various P types including P[3], P[6], P[8], P[9], P[14] in humans and P[1]-[4], P[7], P[9], P[11], P[12], P[19], P[22] and P[24] in various animal species [Gentsch et al., 2005; Matthijnssens et al., 2008b].

Because rotavirus strains exhibit host range restriction (i.e. limited capacity of strains to infect a species other than their natural host), interspecies transmission of rotavirus strains is likely to be a relatively rare event, occurring less frequently compared to gene reassortment events [Ciarlet et al., 1998; Gentsch et al., 2005]. Interspecies transmission is suspected as the mechanism of genetic diversity when strains have greater genome similarity to strains from a different species, as has been observed for G3 strains. Specifically, some G3 rotavirus strains detected in human populations exhibit greater similarity to cat or dog G3 strains compared to other human G3 strains. Reassortment of one or more gene segments has been documented in combination with interspecies transmission between two or more host species [Nakagomi et al., 1990; Khamrin et al., 2006; De Grazia et al., 2008].

Rotavirus G3 strains have been described as having the broadest host range and have been detected in cats, dogs, rabbits, goats, lambs, pigs, horses, cows, mice, birds, monkeys and humans [Tsugawa and Hoshino, 2008; Martínez-Laso et al., 2009]. Animal strains with a G3P[9] genotype are most commonly detected in cat and G3P[3] in both cat and dogs [Hoshino et al., 1982; Birch et al., 1985; Mochizuki et al., 1992; Taniguchi et al., 1994; Nakagomi and Nakagomi, 1989; Martella et al., 2001a, b; Kang et al., 2007]. Human G3 strains with novel P[3] or P[9] genotypes are believed to have originated from direct transmission from cats or dogs to humans as some G3P[3] or G3P[9] strains detected from human are virtually identical to cat or dog strains of the same genotype [Li et al., 1993; Nakagomi and Nakagomi, 2000; Tsugawa and Hoshino, 2008].

Surveillance for the distribution of rotavirus genotypes is critical for monitoring the circulating rotavirus strains (including emerging and vaccine strains), for continued development of new rotavirus vaccines and for evaluating vaccine impact. As vaccine coverage increases, selective pressure could increase the

circulation of uncommon strains, which over time could lead to a reduction in vaccine effectiveness. Surveillance has shown that animal strains can contribute to generating diversity among the human rotavirus population (e.g. bovine G9 rotavirus strains in India; Cunliffe et al., 1997) and could become an increasingly important contributor to diversity due to vaccine selective pressure [Matthijnssens et al., 2009b]. Tracking virus evolution and gaining an understanding of the role that animal rotavirus strains play is important for continued development of prevention strategies to reduce morbidity and mortality associated with rotavirus diarrhea.

The objective of this study was to characterize the diversity of circulating human rotavirus strains and to evaluate the dynamics between human and animal rotavirus strains. The data presented here describe three G3 rotavirus strains obtained from stools of American Indian children. The molecular characterization of each rotavirus strain reveals a unique genetic evolution and provides support for the diversity-generating mechanisms of rotavirus.

MATERIALS AND METHODS

Sample Collection

The oral pentavalent rotavirus vaccine (PRV), RotaTeq[®], (Merck & Co., Inc., Whitehouse, NJ) was licensed in the U.S. in 2006 following the phase-III, multi-center efficacy clinical trial conducted in 11 countries [Vesikari et al., 2006]. Two, U.S.-based sites were the Navajo and Fort Apache Reservations located in the southwest region of the country. Children ages 6–12 weeks from the Navajo and White Mountain Apache tribes were enrolled into trial and randomized to receive three doses of vaccine or placebo at intervals of 28–70 days between doses. Fecal specimens were collected from study participants at or around the time of vaccination and within 14 days of gastroenteritis symptom onset, whenever that occurred through 24 months of age. To describe the distribution of rotavirus genotypes in the community, stool specimens from the placebo group were tested for rotavirus. Rotaviruses from positive specimens were genotyped. Three G3 genotype strains were detected. Because these three G3 strains were associated with an uncommon P type (i.e. P[3] or P[9]), they were further characterized by partially sequencing the rotavirus genome and comparing the genes of each strain to reference human and animal rotaviruses.

RNA Extraction

Double stranded viral RNA was extracted from 10% stool suspensions that were made by adding 0.1 g or 100 ul of the fecal sample to 900 ul of sterile phosphate buffered saline (PBS). Stool suspensions were homogenized by vortexing and clarified by centrifugation for 5 min at 6000 rpm. The supernatant of

each clarified specimen was prepared for extraction according to the MagMAX -96 Viral RNA Isolation KIT protocol (Ambion, Austin, TX) and automated extraction was performed on the KingFisher instrument (Thermo Scientific, Vantaa, Finland).

Rotavirus Detection

Rotavirus in stool specimens was detected by real-time RT-PCR (rRT-PCR) using a modified version of the method described by Freeman et al. [2008]. The rRT-PCR method was adapted in this study for use on the Applied Biosystems 7500 real-time PCR platform. The rRT-PCR assay yields an 87 bp fragment from a highly conserved region of the NSP3 gene. A cycle threshold (Ct) value below 40 indicated that the specimen was positive for rotavirus.

Amplification and Sequencing of Genes

Double-stranded RNA was denatured at 97°C for 5 min and reverse-transcribed and amplified using the Qiagen One-Step RT-PCR Kit (Qiagen, Inc., Valencia, CA). Previously published primers were used for the amplification of genes encoding NSP1 (nt: 1-1568), NSP2 (nt: 1-1059), NSP3 (nt: 1-1084), NSP4 (nt: 1-751), NSP5 (nt: 1-664), VP1 (nt: 1-2037), VP2 (nt: 1-1618), VP4 (nt: 11-887), VP6 (nt: 1-1356) and VP7 (nt: 37-932 [Gentsch et al., 1992; Das et al., 1994; Iturriza-Gómara et al., 2001; Matthijnssens et al., 2006; Kerin et al., 2007; Bányai et al., 2009]). Unpublished primers used for NSP1, VP1, VP2, and VP3 sequence reactions are described in Table I. The target region of each gene was reverse-transcribed at a temperature of 42°C for 30 min followed by deactivation of the reverse-transcriptase at 95°C for 15 min. Amplification of each gene was performed on a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Inc., Foster City, CA) using the following cycle parameters: 30 cycles of 94°C for 30 sec, 42°C for 30 sec, and 72°C for 45 sec, extension at 72°C for 7 min and a final hold at 4°C until RT-PCR products could be prepared for sequence analysis. Amplicons were detected by gel electrophoresis using a 1.5% agarose gel. When one band of the desired length was visible on the gel, RT-PCR products were cleaned using ExoSAP-IT[®] according to manufacturer's instructions (USB Corp., Cleveland, OH) and used directly for sequencing. However, if multiple bands were visible on the gel, the band of the desired length was excised from the gel and purified using the QIAquick Gel extraction Kit protocol (Qiagen) in preparation for sequencing. Cycle sequencing was performed using Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) and the respective primers listed in Table I. Cycle sequencing products were purified using the Big Dye Xterminator Purification Kit (Applied Biosystems, Inc.) and sequenced on the ABI 3130 platform (Applied Biosystems, Inc.).

TABLE I. Unpublished Primers Used for Amplification of the Gene Encoding Rotavirus Proteins

Gene	Primer	Sequence (5'-3')	Polarity	Primer position (nt)
NSP1	JRG32	GGCTTTTTTTTTGAAAAGTCT	+	1-20
VP1	AU-1_VP1F	GGCTATTAAAGCTGTACAATG	+	1-21
	AU-1_VP1R	GCAATCTACTAACCAGTCTTG	-	960-940
VP2	AU-1_VP2F	GGCTATTAAAGGCTCAATGGC	+	1-21
	AU-1_VP2R	GCACTACTGATCTAGCTAAGA	-	1037-1017
VP3	AU-1_VP3F	GGCTTTTAAAGCAGTACGAGT	+	1-21
	AU-1_VP3R	ATATGGAGTGTCAAATGGATCC	-	991-970
	JRG256	GGCTATTAAAGCAGTACCAGTAGTGTGT	+	1-28
	M_VP3R	TGCGGAAATGGGAAGYTCTAAATCC	-	1234-1207

Sequence Analysis and Genotyping

Nucleotide sequences were assembled using the software program, Sequencher (Gene Codes Corporation, Inc., Ann Arbor, MI) and compared to reference strains in the NCBI (National Center for Biotechnology Information) GenBank database using BLAST (Basic Local Alignment Search Tool) [Altschul et al., 1990]. Phylogenetic analysis of strains was carried out using MEGA version 4.0 [Tamura et al., 2007] to align strains at the nucleotide level and to create dendrograms for each gene using the neighbor-joining method [Saitou and Nei, 1987]. Distance matrices were constructed using MEGA at the nucleotide and amino acid levels to evaluate percentage identity between strains.

GenBank Accession Numbers

Nucleotide sequences of all sequenced gene segments were deposited in GenBank with the following accession numbers: JF804984-JF805015.

Human Subjects

Informed consent was obtained from parents or caregivers of children who were enrolled into the vaccine trial. Institutional Review Boards at Johns Hopkins Bloomberg School of Public Health, Centers for Disease Control and Prevention, the Navajo Nation and the Phoenix Area Indian Health Service approved this study as did the tribes.

RESULTS

Three Group A rotavirus strains referred to as strains 6212, 6235, and 0537 with uncommon genotype results were detected in stool specimens. Of these, strain 6212 was detected in a non-diarrheal stool specimen while strains 6235 and 0537 were detected in stools from children with gastroenteritis. The specimens associated with these strains were collected between March 2002 and January 2003 from children aged 3 to 6 months.

An attempt was made to obtain a complete or partial sequence all 11 rotavirus genes from each of the three strains. However, the VP2 from strain 6235 could

not be amplified after multiple attempts with various primer combinations and PCR parameters.

Genotype Analysis

The full-genome classification scheme proposed by Matthijnssens et al. [2008a] was applied for genotype assignment of strains (Table II). Genotyping results are presented only for VP4, VP7, VP6, and NSP4 in this manuscript. Genotypes were determined by adhering to the following steps: (1) alignment of specimen nucleotide sequences with reference strains from GenBank that are representative of at least one genotype for all sequenced genes, (2) pairwise comparisons of gene sequences from specimens with reference strains of the same and different genotypes, (3) assignment of genotypes using the percent nucleotide identity cut-off values [Matthijnssens et al., 2008a], and (4) generation of phylogenetic trees that included the three strains described here and a variety of reference strains to verify genotype classification. For partially sequenced genes the following requirements had to be met to assign a genotype: at least 50% of the open reading frame (ORF) and at least 500 nucleotides of the ORF had to be sequenced and the percentage identity cutoff had to be increased by two percentage points [Matthijnssens et al., 2008a]. Complete nucleotide sequences were used for genotyping except for the longer genes (VP1-VP3) and VP4 where a ~1kb 5' fragment was sequenced.

Except for VP1, sequenced genes of strain 6212 yielded an identical genotype to dog (A79-10, CU-1 and K9), cat (Cat97) and human strains (HCR3A and Ro1845) with the following genotype: G3-P[3]-I3-R1-C2-M3-A9-N2-T3-E3-H6. Excluding VP2, which could not be sequenced, genes of strain 6235 yielded a similar genotype as 6212 (G3-P[3]-I2-R1-Cx-M3-A9-N2-T3-E3-H6) except for VP6, which genotyped as I2. Compared to other G3P[3] strains, including dog (K9 and A79-10), cat (Cat97) and human (HCR3A and Ro1845) strains, sequenced genes of 6212 and 6235 shared around 90% or greater nucleotide identity for genes of the same genotype.

Genotypes for strain 0537 were assigned to all genes as follows: G3-P[9]-I2-R2-C2-M2-A3-N2-T1-E2-H3. Even though strain 0537 is classified by VP4 and

TABLE II. Classification and Comparison of Genotypes for Genes From G3P[3] and G3P[9] Strains With Reference Rotavirus Strains

Genes encoding	Genotypes identity (No.) ^a	% nt identity of 6212 to:		% nt identity of 6235 to:		Genotype of:		% nt identity of 0537 to:	
		G3P[3] strains (max identity)	(Prototype strain) ^b	G3P[3] strains (max identity)	(Prototype strain) ^b	G3P[9] strain ^d	(Prototype strain) ^b		
NSP1	A (16)	A9	83(RRV)	93-96(Ro1845)	83(RRV)	90-93(A79-10)	A3	94(AU-1)	85-94(RF, AU-1)
NSP2	N (6)	N2	83(DS-1)	95-96(K9)	83(DS-1)	91-92(K9)	N3	87(DS-1)	84-95(Cu-1, B4106)
NSP3	T (8)	T3	87(AU-1)	94-96(A79-10, K9)	86(AU-1)	94-95(A79-10, HCR3A)	T3	90(Wa)	88-90(Dhaka12-03, Dhaka25-02)
NSP4	E (12)	E3	83(AU-1)	94-97(HCR3A)	84(AU-1)	91-93(A79-10, Ro1845)	E3	85(DS-1)	84-97(SA11[H96], PA169)
NSP5	H (8)	H6	93(RRV)	96-98(K9)	93(RRV)	95-96(HCR3A)	H3	94(AU-1)	90-98(B4106, Cat2)
VP1	R (6)	R3	97(Wa)	79-80(A79-10, K9)	98(Wa)	79-80(A79-10, K9)	R3	85(DS-1)	91-95(B4106, NCDV)
VP2	C (6)	C2	91(DS-1)	94-95(A79-10)	—	—	C3	84(DS-1)	84-92(DS-1, Se584)
VP3	M (7)	M3	83(AU-1)	94-97(HCR3A)	82(AU-1)	91-92(A79-10, Cat97)	M3	85(DS-1)	89-91(BRV033, NCDV)
VP4	P (31)	P[3]	77(RRV)	93-96(K9)	77(RRV)	93-96(K9)	P[9]	95(AU-1)	94-98(FRV1/02-92, Hun2)
VP6	I (13)	I3	87(AU-1)	93-96(A79-10, RUBV3)	87(DS-1)	79-80(CU-1)	I3	87(DS-1)	87-96(SA11[H96], 22R)
VP7	G (23)	G3a ^e	83(RRV)	93-96(HCR3)	84(RRV)	89-91(Ro1845)	G3b ^e	91(AU-1)	77-95(B4106, PA158-96)

^aGenotype code and no. = number of genotypes per gene [Matthijnsens et al., 2009a].
^bCa/A79-10, Ca/K9, Fe/Cat97, Hu/HCR3A, Hu/Ro1845.
^cGenotype of prototype strains as determined by Matthijnsens et al. [2008a].
^dHu/AU-1.
^eG3a, G3b designation by Matthijnsens et al. [2008a].

VP7 as AU-1-like, only two other typed genes (NSP1 and NSP5) were also classified as AU-1-like with percentage identities to AU-1 of 85.1–94.2% and 94–97.9%, respectively. The sequence of NSP3 exhibited around 90% identity with Wa-like strains, while sequences of the genes encoding NSP4, VP1, VP3, and VP6 shared between 85–95% identity with DS-1-like strains.

VP7 Gene Sequence Analysis

For VP7 sequences, strains 6212 and 6235 were highly similar to each other (nucleotide [nt]: 89.2%, amino acid [aa]: 92.1%) as well as to other G3 strains of dog (nt: 81–95%, aa: 91–97%), cat (nt: 78–93%, aa: 92–96%), human (nt: 76–98%, aa: 83–98%), lapine (nt: 87–89%, aa: 89–92%), porcine (nt:78–81%, aa:87–88%) and simian origins (nt:80–84%, aa:89–92%) (Table III). Strains 6212 and 6235 clustered with and were most closely related to human G3 strains, HCR3 and 7717–1042 (nt: 96.3%, 98.1%, aa: 98.3%, 98.3%) [Li et al., 1994; Laird et al., 2003] (Appendix, Fig. I).

Both 6212 and 6235 strains clustered within G3 lineage III [Martínez-Laso et al., 2009], which in this case is constructed solely from dog, cat and human G3P[3] strains; however, strains 6235 and 7717–1042 formed their own distinct branch within this lineage. For lineages I, II, III, and IV strains, the nucleotide and amino acid percentage identities with 6212 and 6235 are: (I) 76–81%, 83–90%; (II) 80–83%, 89–93%; (III) 89–98%, 92–98; (IV) 86–89%, 89–92%. Common human G3 strains (e.g. M, P, and YO) predominantly cluster in lineage I (data not shown) and have around 79% nucleotide and 90% amino acid homology with strains 6212 and 6235.

The VP7 nucleotide sequence of strain 0537 is also most closely related to G3 strains of dog (nt: 78–80%, aa: 89%), cat (nt: 78–94%, aa: 89–96%), human (nt: 77–95%, aa: 88–95%), porcine (nt: 84–85%, aa: 91–92%) and simian origin (nt: 78–80%, aa: 89–90%) with the highest nucleotide percentage identity observed with human reference strain PA158/96 (nt: 95.4%, aa: 95.2%) [De Grazia et al., 2008]. Strain 0537 clustered with G3 lineage I, which consisted primarily of human strains, including uncommon human strains AU-1, CC425 and KC814, and several animal strains with identity values ranging from 85 to 95% (nt) and 90–96% (aa).

VP4 Gene Fragment Sequence Analysis

Based on percent nucleotide identity of the 5' VP4 fragment, strains 6212 and 6235 belong to genotype P[3] and P[9] for strain 0537 (Table IV). All but two (RUBV3 and RRV) of the P[3] reference strains represented in Table IV have percentage identity values with strains 6212 and 6235 that are above the genotype determination cutoff of 80% (% nt identity above cutoff: 93–97%, below cutoff: 76%). When both P[3] strains were compared with the remaining P

TABLE III. Comparison of Nucleotide and Amino Acid Identity for VP7 Between Identified G3P[3] and G3P[9] Strains and Various Reference Rotavirus Strains

Host species/Strain ^a	Genotype (G3 lineage) ^b	Hu/6212		Hu/6235		Hu/0537	
		nt (%)	aa (%)	nt (%)	aa (%)	nt (%)	aa (%)
Hu/6212	G3 (III)	—	—	89.2	92.1	78.9	88.3
Hu/6235	G3 (III)	89.2	92.1	—	—	78.2	87.9
Hu/0537	G3 (I)	78.9	88.3	78.2	87.9	—	—
Hu/Wa ^c	G1	71.7	77	72.6	76.6	71.7	77.8
Hu/DS-1	G2	70	72	70	70.7	70.7	72
Hu/TB-Chen	G2	70.2	72.4	70	71.1	71	71.5
Ca/A79-10	G3 (III)	95.8	97.9	91	93.3	78.6	89.1
Ca/CU-1	G3 (III)	95.2	97.9	90.2	93.3	78.7	89.1
Ca/K9	G3 (III)	94.1	96.7	91.3	94.1	78.1	89.1
Ca/RV52-96	G3 (II)	81.1	91.6	81.9	91.2	80.5	89.1
Fe/Cat2	G3 (I)	78.2	90	79.2	90	94.6	96.7
Fe/Cat97	G3 (III)	93.2	96.7	89.6	92.1	78.9	89.5
Hu/02-92	G3 (I)	77.7	84.9	76.8	83.7	88.4	90
Hu/7177-1042	G3 (III)	89.2	92.9	98.1	98.3	78.1	88.7
Hu/AU-1	G3 (I)	79	89.1	78.9	87.9	90.9	94.6
Hu/B4106	G3 (IV)	86.8	92.1	86	90.4	77.1	90
Hu/CC425	G3 (I)	77.9	89.5	79.4	89.5	94	95
Hu/HCR3	G3 (III)	96.3	98.3	90.1	93.7	78.5	88.7
Hu/PA260-97	G3 (II)	81.2	91.2	82.3	90.8	80.1	88.7
Hu/PAI58/96	G3 (I)	80	89.1	78.9	89.1	95.2	95.4
Hu/P	G3 (I)	79.1	90.2	80.6	91.3	91.6	94.4
Hu/Ro1845	G3 (III)	94	96.2	91.3	93.7	78.7	88.7
Lap/30-96	G3 (IV)	89	92.5	86.9	89.5	78.7	88.3
Po/4F	G3 (I)	79.4	88.7	78.9	88.7	85.4	92.5
Po/A131	G3 (I)	80.4	87.4	81.1	87	84.6	91.2
Si/RRV	G3 (II)	83.2	92.9	84.2	92.1	80.7	89.5
Si/SA11[H96]	G3 (II)	82.4	91.2	80	89.5	78.2	90
Hu/Hochi	G4	71.1	73.6	71.4	72.8	74.9	74.5
Po/OSU	G5	74.4	81.6	75.1	81.2	75.5	82.8
Bo/RF	G6	73	81.6	74	80.3	73.8	81.2
Av/Ch2	G7	63.6	63.2	62.8	62.3	62.7	61.5
Hu/69M	G8	72.2	81.6	73	79.1	73.6	81.6
Hu/Wi61	G9	75.6	82.4	74.7	81.2	77.7	81.2
Bo/61A	G10	73.3	79.5	73.6	79.1	73.8	78.7
Po/YM	G11	76.2	84.1	76	82.4	75.9	85.4
Hu/Dhaka12-03	G12	72.8	77	73	74.9	70.4	75.7
Eq/L338	G13	75.6	80.8	76.6	78.2	74.8	77.8
Eq/CH3	G14	77.8	82.8	79.2	82.8	78.9	82
Bo/Hg18	G15	71	73.6	71.1	73.2	68.8	73.6
Mu/EW	G16	74.9	86.6	75.7	83.7	74.4	84.1
Av/Ty-1	G17	66.5	66.5	66.8	66.1	64.2	64.4
Av/PO-13	G18	65.3	64.4	64.7	63.2	62.8	62.3
Av/Ch-1	G19	64.4	62.8	63.6	61.1	62	60.3
Hu/Euc534	G20	74.6	87.5	75.3	85.4	73.4	84.2
Bo/AzuK-1	G21	70.8	75	71.3	73.7	71.3	75
Av/Tu-1E10	G22	64.1	63.3	63.9	62.9	62.8	60.8
Av/Phea14246 Hun-08	G23	66.7	63.8	65.7	62.9	65.3	62.1

^aHost species abbreviations are as follows: Hu = human, Ca = canine, Fe = feline, Lap = lapine, Po = porcine, Si = simian, Bo = bovine, Av = avian, Eq = equine, Mu = murine.

^bG3 lineages as assigned by Martínez-Laso et al. [2009].

^cGenBank accession numbers for reference strains: Wa (K02033), DS-1 (EF672581), TB-Chen (AY787646), A79-10 (EU708939), CU-1 (EU708917), K9 (EU708928), RV52-96 (AF271090), Cat2 (EU708961), Cat97 (EU708950), 02-92 (D86264), 7177-1042 (AJ488587), AU-1 (D86271), B4106 (AY456382), CC425 (AJ311738), HCR3 (L21666), PA260-97 (EF442733), PAI58/96 (EU076907), P (EF672602), Ro1845 (EU708895), 30-96 (AF528204), 4F (L10360), A131 (L35055), RRV (M21650), SA11[H96] (DQ838620), Hochi (AB012078), OSU (X04613), RF (X65940), Ch2 (X56784), 69M (EF672560), Wi61 (EF672623), 61A (X53403), YM (M23194), Dhaka12-03 (DQ146665), L338 (D13549), CH3 (D25229), Hg18 (AF237666), EW (U08430), Ty-1 (S58166), PO-13 (D82979), Ch-1 (AB080738), Euc534 (EU805775), AzuK-1 (AB454421), Tu-1E10 (EU486972), Phea14246-Hun-08 (FN393054).

types, the nucleotide and amino acid identities ranged from 50–74% and 28–65%.

Both P[3] strains (6212 and 6235) clustered within the sub-cluster of P[3] reference strains except for RUBV3 and RRV, which formed a separate P[3] sub-cluster (see Supporting information for Appendix A,

Figure II). The gene encoding VP8* of strains 6212 and 6235 share a high percentage nucleotide and amino acid identity with each other (nt: 96.4%, aa: 94.2%) as well as with P[3] strains of dog (nt: 93–97%, aa: 88–95%), cat (nt: 94, aa: 90%) and human origin (nt: 93–96%, aa: 87–97%). Strains 6212 and 6235

TABLE IV. Comparison of Nucleotide and Amino Acid Identity for VP4 Between Identified G3P[3] and G3P[9] Strains and Various Group A Rotavirus

Host species/Strain ^a	P[Genotype no.]	Hu/6212		Hu/6235		Hu/0537	
		nt (%)	aa (%)	nt (%)	aa (%)	nt (%)	aa (%)
Hu/6212	P[3]	—	—	96.4	94.2	56.3	40.9
Hu/6235	P[3]	96.4	94.2	—	—	57.3	41.6
Hu/0537	P[9]	56.3	40.9	57.3	41.6	—	—
Bo/RF ^b	P[1]	73.3	61.3	72.3	62	60.5	46.7
Si/SA11[H96]	P[2]	73.9	65.7	72.9	65	57.8	43.8
Fe/Cat97	P[3]	94.7	92	94.8	90.5	58.4	46
Ca/RV198-95	P[3]	93.7	91.2	93.1	88.3	58.2	45.3
Bo/RUBV3	P[3]	76.3	65.7	75	62	57.4	45.3
Ca/RV52-96	P[3]	95	90.5	94.8	89.1	57.1	43.8
Ca/A79-10	P[3]	94.7	90.5	94.5	90.5	56.7	43.8
Si/RRV	P[3]	76.9	65.7	76.3	66.4	59	43.1
Ca/CU-1	P[3]	93.3	88.3	93.5	88.3	57.3	43.1
Hu/PA260-97	P[3]	95.4	91.2	95.2	89.8	57.1	43.1
Hu/PAI58/96	P[3]	58.2	43.5	59.2	46.4	94.6	94.2
Hu/Ro1845	P[3]	93.7	91.2	92.7	86.9	57.3	42.3
Hu/HCR3A	P[3]	96.9	97.1	96	94.9	57.3	41.6
Ca/K9	P[3]	97.3	95.6	96.4	94.2	56.9	40.9
Hu/DS-1	P[4]	59.9	43.1	59.4	41.6	55.7	39.4
Bo/UK	P[5]	59.4	40.1	59.2	38	52.9	40.1
Po/Gottfried	P[6]	60.7	44.5	59.9	43.8	52.9	38
Po/OSU	P[7]	73.1	59.9	71.9	57.7	59.7	46
Hu/KU	P[8]	59.4	40.1	59.2	38	52.9	40.1
Hu/Hun2	P[9]	57.3	42.3	57.8	43.1	98.5	97.8
Hu/AU-1	P[9]	57.4	42.3	58.4	43.1	95.6	94.9
Hu/CC425	P[9]	62.5	49	63.6	49	94.7	92
Hu/02-92	P[9]	56.9	41.6	57.8	42.3	94.3	92.7
Fe/Cat2	P[9]	57.3	41.6	58.2	42.3	95.2	93.4
Fe/FRV1	P[9]	57.4	43.1	58	43.8	94.3	92.7
Hu/69M	P[10]	69.7	59.9	67.9	56.9	55.9	42.3
Bo/B223	P[11]	44.5	28.5	43.7	29.9	43.1	25.5
Eq/H-2	P[12]	67.4	53.3	66.4	54.7	58.2	42.3
Po/MDR-13	P[13]	64.1	51.1	63.5	49.6	53.8	36.5
Hu/PA169	P[14]	55.9	43.1	57.3	43.8	74.8	67.9
Ov/Lp14	P[15]	69.1	56.2	69.8	56.2	56.9	39.4
Mu/EW	P[16]	59.2	44.5	59.5	44.5	53.6	40.9
Av/PO-13	P[17]	50	29.2	49.6	29.2	48.7	30.7
Eq/L338	P[18]	73.3	59.9	72.7	60.6	59.7	45.3
Hu/Mc323	P[19]	65.5	46.7	65.3	45.3	53.8	40.1
Mu/EHP	P[20]	67.4	59.1	67	58.4	54.6	43.1
Bo/Hg18	P[21]	67.7	51.1	68.1	51.8	55.5	37.2
Lap/160/01	P[22]	56.3	44.5	57.4	46	71.2	60.6
Po/A34	P[23]	72.1	59.1	71.6	59.9	57.6	39.4
Si/TUCH	P[24]	69.8	59.1	70	59.1	56.5	40.9
Hu/Dhaka6	P[25]	60.7	52.6	60.5	52.6	70.4	57.7
Po/134/04-15	P[26]	66	51.1	65.1	49.6	55.7	42.3
Po/CMP034	P[27]	62.2	48.5	60.8	46.3	53.4	34.3
Hu/Ecu534	P[28]	65.9	61.2	65.5	62	55.5	51.2
Bo/AzuK-1	P[29]	54.1	40.3	54.1	38.8	46.8	28.7
Av/Ch-2G3	P[30]	53	35.7	52.6	33.3	50.9	34.9
Av/Ch-661G1	P[31]	50.1	32.6	50.1	32.6	46.1	34.1

^aPreviously unspecified host species abbreviations are as follows: Ov = ovine.

^bGenBank accession numbers for reference strains: RF (U65924), SA11[H96] (DQ841262), Cat97 (EU708948), RV198-95 (AF339843), RUBV3 (EF200553), RV52-96 (AF339844), A79-10 (EU708937), RRV (M18736), CU-1 (EU708915), PA260-97 (EF442735), PAI58/96 (GU296427), Ro1845 (EU708893), HCR3A (EU708904), K9 (EU708926), DS-1 (EF672577), UK (M21014), Gottfried (M33516), OSU (X13190), KU (M21014), Hun2 (AJ488137), AU-1 (D10970), CC425 (AJ311734), Cat2 (EU708959), FRV1 (D10971), 02-92 (AB008289), 69M (M60600), B223 (D13394), H-2 (L04638), MDR-13 (L07886), PA169 (D14724), Lp14 (L11599), EW (U08429), PO-13 (AB009632), L338 (D13399), Mc323 (D38052), EHP (U08424), Hg18 (AF237665), 160/01 (AF526376), A34 (AY174094), TUCH (AY596189), Dhaka6 (AY773004), 134/04-15 (DQ061053), CMP034 (DQ534016), Ecu534 (EU805773), AzuK-1 (AB454420), Ch-2G3 (EU486956), Ch-661G1 (EU486962).

clustered most closely with K9 and HCR3A [Fulton et al., 1981].

The 0537 strain clustered with the genotype P[9] strains, while forming its own distinct P[9] sub-cluster with human reference strain Hun2 (G type: G3, with

which it shared the highest percentage identity of 98.5%). Of the P[9] reference strains in Table IV, nucleotide and amino acid percentage identities range from 94.3–98.5% (nt) and 92–97.8% (aa) for strain 0537. The VP8* nucleotide sequence of strain 0537 is

most closely related to cat (Cat2, FRV1—nt: 95%, aa: 93%) and human (Hun2, AU-1, 02-92, K8—nt: 94–98%, aa: 92–97%) P[9] strains with the highest percentage identity observed with the human reference strain Hun2 (nt: 98.5%, aa: 98%) [Bányai et al., 2009]. For P types other than P[9], the nucleotide and amino acid percentage identity ranged from 43–71% and 30–60%.

VP6 Gene Sequence Analysis

Strain 6212 was assigned to the I3 genotype while strains 6235 and 0537 both typed as I2 (see Supporting information for Appendix, Table I). Percent identity of strains 6212, 6235, and 0537 with I2 and I3 reference strains ranged from 87–96% while the identities with other “I” types ranged from 68–82%. Strains 6235 and 0537 clustered with the genotype I2/DS-1-like reference strains while strain 6212 clustered with the genotype I3/AU-1-like strains (see Supporting information for Appendix, Figure III). Strain 6235 and 0537 shared the highest nucleotide identity with bovine strains, 22R and RUBV3, with 92.9% and 95.8% identity, respectively (see Supporting information for Appendix, Table I) [Matsumura et al., 2002, Ghosh et al., 2007]. Human strain, US8720, was detected during U.S. surveillance between 1998–1999, clustered with the genotype I2/DS-1 strains and shared between 92–94% and 97–99% identity on the nucleotide and amino acid levels with strains 6235 and 0537.

Strain 6212 was most closely related to human strain Ro1845 with a percentage identity of 95.8%. Reference strains with percentage nucleotide identity values above the cut-off value are indicated in bold (see Supporting information for Appendix, Table I).

NSP4 Gene Sequence Analysis

Strains 6212 and 6235 clustered directly with an E3 sub-cluster constructed primarily of dog and cat strains. Strains belonging to either of the E2 and E3 genotypes formed two distinct sub-clusters within the greater E2 or E3 clusters (see Supporting information for Appendix, Figure IV).

Strains 6212 and 6235 had the highest percentage identity with animal and human E3 type strains with nucleotide values ranging from 92–97% for strain 6212 and 92–93% for strain 6235 (see Supporting information for Appendix, Table II). Strain 6212 clustered directly with HCR3A with 97.4% and 94.7% nucleotide and amino acid identity. Strain 6235 and dog strain A79-10 shared the highest percentage identity at 93.1% (nt) and 86.2% (aa). Strain 6235 did not cluster directly with A79-10, although both strains were located within the same E3 sub-cluster. Reference strains of other genotypes had 50–78% and 27–62% nucleotide and amino acid identity with both strains 6212 and 6235.

Strain 0537 shared the highest nucleotide percentage identity with two G6P[14] human reference

strains, Hun5 and PA169 (91.7% to 97.4%) forming a distinct E2 sub-cluster. Percentage identity values with reference strains of other genotypes ranged from 49–80% for nucleotides and 25–58% for amino acids (see Supporting information for Appendix, Table II).

DISCUSSION

Over a decade ago, Li et al. [1993, 1994], characterized an unusual rotavirus strain detected in the stool of a clinically asymptomatic infant in the United States. This strain, HCR3, genotyped as G3P[3] and was determined to be closely related to dog and cat rotavirus strains. To our knowledge, HCR3 is the only G3P[3] strain detected in a human specimen from the U.S. that has been described in the literature to date. Human G3P[3] strains detected in other countries (e.g., Ro1845 from Israel [Aboudy et al., 1988]) are also highly similar to G3P[3] dog strains such as A79-10, K9 and CU-1 and cat strain Cat97 [Tsubawa and Hoshino, 2008]. In the U.S., rare strains with a G3P[9] genotype are detected approximately once annually by routine surveillance [Griffin et al., 2000; Hull et al., 2011]. Of the genotyped rotavirus strains detected in fecal samples of Navajo and White Mountain Apache children, two typed as G3P[3] (strains 6212 and 6235) and one as G3P[9] (strain 0537).

In this study, we demonstrated that the G3P[3] strains, 6212 and 6235, were highly similar to each other and to other G3P[3] strains of cat and dog origin, except for VP1 of both strains and VP2 and VP6 of strain 6235. VP1 of both strains typed as R1 with > 96% nucleotide similarity to the human Wa strain, while VP6 of 6235 was most similar to cow, human and simian strains with the I2 genotype (e.g. 22R, RF, RUBV3, US8720, PAI58/96, RRV). For NSP1-5, VP3, VP4 and VP7, strains 6212 and 6235 shared greater than 90% nucleotide identity with G3P[3] strains (A79-10, K9, CU-1, Cat97, HCR3A and Ro1845). Together, these results suggest that (1) both strains may have evolved from cat or dog G3P[3] strains, (2) VP1 of both strains 6212 and 6235 underwent a reassortment event with a human strain of the R1 genotype and (3) VP6 of strain 6235 underwent a reassortment event with a cow, human or simian strain of the I2 genotype. These results further suggest that the genotypes associated with cat and dog rotavirus strains do not preclude them from infecting humans.

The G3P[3] strains have caused both symptomatic and asymptomatic infections of cats, dogs and humans despite a high percentage identity between strains associated with these different clinical symptoms [Tsubawa and Hoshino, 2008]. In this study, strain 6212 was detected from the stool of an asymptomatic child while strain 6235 was detected from a child with gastroenteritis. These results support the variable pathogenesis of G3P[3] infections; however, it is unknown whether the child from whom strain 6212

was isolated had a previous rotavirus infection that could have provided some protection against symptoms.

The G3P[9] strain (0537) yielded divergent genotypes for genes encoding NSP2-NSP4, VP1-VP3 and VP6 compared to previously described G3P[9] reference strains (AU-1 and Cat2) [Birch et al., 1985; Nakagomi et al., 1987]. The T1 genotype for NSP3 has been observed in human Wa-like strains and in bovine and porcine strains. For NSP2, NSP4, VP1-VP3 and VP6, the N2, E2, R2, C2, M2 and I2 genotypes are classified as DS-1-like, which are the same genotypes for bovine strains (e.g., RF). The H3 genotype for NSP5 is associated with the prototype G3P[9] human strain, AU-1, and with bovine and feline strains (e.g. B4106 and Cat2). Interestingly, strain 0537 shares the same combination of genotypes for all genes except for VP7 with human G6P[9] strain, Se584, that was detected by U.S. surveillance [Griffin et al., 2002]. Strain 0537 could represent a single segment reassortant of a Se584-like strain.

G3P[3] rotavirus strains have been documented in bovine, dog, goat, cat, human, and simian populations [Aboudy et al., 1988; Mackow et al., 1988; Li et al., 1993; Taniguchi et al., 1994; Martella et al., 2001a, b; Lee et al., 2003; Estes and Kapikian, 2007; Ghosh et al., 2007]. Detection of G3P[3] strains in humans is relatively rare, although several strains have recently been characterized. The human G3P[3] strains that have been detected and characterized (HCR3A, HCR3B, CMH222, PA260/97, Ro1845, and CRI33594) have a high degree of homology to the dog and cat G3P[3] strains [Li et al., 1993, 1994; Santos et al., 1998; Khamrin et al., 2006; Banerjee et al., 2007; De Grazia et al., 2007; Tsugawa and Hoshino, 2008]. Strains 6212 and 6235 also exhibit a high percentage nucleotide identity to cat and dog G3P[3] strains for all sequenced genes except for VP1 and VP6, which genotyped as R1 for both strains and I2 for strain 6235, respectively. Based on genotype and nucleotide identity results, strains 6212 and 6235 could have originated from cats or dogs followed by reassortment events with human strains bearing the R1 genotype for VP1 and the I2 genotype for VP6 (strain 6235 only). There are multiple possible explanations as to how strains 6212 and 6235 originated; however, there is not enough evidence to predict the timing or sequence of events leading to the creation of strains 6212 and 6235. Detection of strains 6212 and 6235 in human stools is consistent with the increasing number of reports on atypical rotaviruses in humans that likely originated from other species. G3 strains have been detected in various socioeconomic settings and multiple geographic locations including Australia, China, Israel, Italy, Japan, Spain, Thailand, the U.S., and others, which provides evidence that these strains do not appear to be related to a particular community or development setting.

Since the initial detection of G3P[9] human prototype strain AU-1 from Japan, G3P[9] rotavirus strains

have been detected more frequently in human populations compared to G3P[3] strains [Nakagomi et al., 1987]. Human G3P[9] strains have been detected in several countries including China, Hungary, Israel, Italy, Japan, Spain, Thailand, and the United States [Nakagomi et al., 1987; Nakagomi et al., 1992; Iizuka et al., 1994; Gollop et al., 1998; Cao et al., 1999; Griffin et al., 2002; Sánchez-Fauquier et al., 2006; Khamrin et al., 2007; De Grazia et al., 2008; Wang et al., 2009; Bányai et al., 2003]. Cat G3P[9] rotavirus strains (Cat2 and FRV1) have also been detected in Australia and Japan [Birch et al., 1985; Mochizuki and Yamakawa, 1987]. While there are some cat and human G3P[9] strains exhibit a high degree of nucleotide identity only with each other (e.g. AU-1 and FRV1 [Nakagomi and Nakagomi, 1989; Iizuka et al., 1994]), there are G3P[9] strains of both human (CC425, KC814, L261,PCP5, PA151, Ro strains, 02/92) and cat (Cat2) origin that are highly similar to strains of bovine and dog origin suggesting that these strains have likely evolved from multiple reassortment events with strains from different host species [Mochizuki et al., 1992; Gollop et al., 1998; Cao et al., 1999; Griffin et al., 2002; Nakagomi and Nakagomi, 2002; Tsugawa and Hoshino, 2008; Wang et al., 2009]. The genotyping and nucleotide identity results for strain 0537 demonstrate that this strain also likely evolved by multiple gene reassortment events between strains from different species.

Data from a previous rotavirus vaccine trial conducted from 1992 to 1994 in American Indian children revealed that G3 was the predominant circulating rotavirus serotype during this trial [Santosham et al., 1997]. For the purpose of calculating vaccine efficacy, only the G type of each rotavirus strain detected from a diarrhea episode was obtained. Retrospectively genotyping the remaining G3 strain genes would be interesting to determine the proportion of these strains that exhibit the P[3] or P[9] genotypes.

Since 1999, U.S.-based surveillance for rotavirus diarrhea and serotypes has detected relatively few to no uncommon strains that are similar to strains 6212, 6235, and 0537. Detection of three uncommon G3 strains in a relatively small collection of samples originating from a geographically isolated location suggests that these populations and locations have a different rotavirus epidemiology than cities where rotavirus surveillance is conducted. Exposure of humans to circulating animal rotavirus strains may be greater in rural locations or contact between animals and humans may occur more frequently.

In the PRV trial [Vesikari et al., 2006], stool specimens were only collected from children enrolled into the trial. No animal specimens were collected during this period to try to identify human rotavirus genes that had reassorted with circulating animal strains or to assess the occurrence of interspecies transmission between humans and animal populations, specifically cats or dogs. For future studies, collecting cat and dog

fecal specimens would be of interest to survey the frequency of interspecies transmission, to monitor the co-evolution of human and animal rotavirus strains and to identify human/animal reassortant viruses.

As of 2006, two rotavirus vaccines (RotaTeq[®], Merck & Co., Inc and Rotarix[®], GlaxoSmithKline Biologicals, Rixensart, Belgium) have been licensed in the U.S. Continued surveillance of circulating rotavirus strains will remain essential for detection of possible reassortants between vaccine and wild-type (human or animal) strains and to measure the relative frequency of detection for any of these reassortant strains.

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