Determination of the G and P Types of Previously Nontypeable Rotavirus Strains from the African Rotavirus Network, 1996–2004: Identification of Unusual G Types

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A total of 215 nontypeable rotavirus samples collected from children <5 years of age by members of the African Rotavirus Network were characterized using reverse-transcription polymerase chain reaction analysis and sequencing. The most predominant strain identified was P[8]G1 (46.9%). Genotypes P[8]G10, P[8]G8, P[6]G8, and P[7]G5 were also detected at frequencies varying from 0.5% to 2.3%. This study suggests that reassortment of unusual G types into a background of globally common genotype P[8] strains may be a major mechanism of generating rotavirus diversity. Nucleotide substitutions at the P[8], P[6], and G1 primer binding sites accounted for the failure to type these strains initially. Hence, these findings highlight the need for regular evaluation of rotavirus genotyping methods.

Group A rotaviruses are the most important etiological agents of severe diarrhea in infants and young children worldwide. Globally, ~600,000 children die every year from rotavirus infection, and most deaths are in developing countries [1]. Knowledge of strain distribution has become of increased importance because new rotavirus vaccines have just been licensed, and it will be critical to assess how effective these are against the diversity of strains in circulation.

Rotavirus is classified as a genus within the *Reoviridae* family and contains a genome of 11 segments of double-stranded RNA that encode 6 structural (VP1–VP4 and VP6–VP7) and 6 nonstructural (NSP1–NSP6) proteins [2]. The 2 outer capsid proteins, VP4 and VP7, define serotypes P (protease-sensitive protein VP4), encoded by gene segment 4, and G (glycoprotein VP7), encoded by gene segment 7, 8, or 9, depending on the strain. Both proteins elicit neutralizing antibody responses and form the basis for classifying group A rotavirus into P and G serotypes and genotypes [2].

To conduct rotavirus strain surveillance for the common serotypes in circulation, both serologic and molecular methods have been used. These include im-

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Members of the African Rotavirus Network are listed at the end of the text.

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munoassays using monoclonal or polyclonal antibodies [3–5] and multiplex reverse-transcription (RT) polymerase chain reaction (PCR)–based assays supported by nucleotide sequencing [6–8].

With a combination of these methods, a total of 11 G and 12 P genotypes have been recovered from humans [9]. Serotypes G1–G4 and G9 are frequently associated with diarrhea in children and, except for serotype G9, they have become prime targets for vaccine development [10]. Rotavirus serotypes P1A[8], P2A[6], and P1B[4] are the globally most common P types that infect humans and P1A[8] is found in licensed vaccines. Genotype P[8] is almost always associated with VP7 types G1, G3, G4, and G9, and genotype P[4] is usually associated with G2 [10]. Although the ability of rotavirus genes to segregate independently could in theory lead to numerous combinations of known G and P serotypes, only 5 strains—P1A[8]G1; P1A[8]G3; P1A[8]G4; P1A[8]G9 and P1B[4]G2—are thought to be globally common. However, other uncommon G and P types (eg, G5, G6, G8, G10, G12, P[9], P[11], and P[14]) have been reported in a variety of locations worldwide [10–12]. Some of these, such as P[6]G8 in Malawi [13], P[8]G5 in Brazil [14], and P[11]G10 in India [15], are important causes of diarrhea in countries where they are found. The impact of these unusual types on rotavirus vaccine development is yet to be determined, but such strains may represent an important route for introduction of new P or G genes into a naive human population via reassortment events [9, 10, 16].

Although monoclonal antibody (MAb) enzyme immunoassay (EIA) serotyping and RT-PCR genotyping assays have been invaluable in defining the importance of individual rotavirus G and P types, recent challenges to the effectiveness of these typing assays have been identified. For example, with MAb-EIA serotyping, ~20%–30% or even more of samples cannot be typed owing to low antigen levels or antigenic variation in MAb binding sites, and some strains cross-react with >1 serotype-specific MAb [5, 17, 18]. MAb-EIA methods for P serotyping have been developed but are cross-reactive and not routinely available [19, 20]. On the other hand, genotype-specific PCR primers have increasingly failed to amplify the VP7 and VP4 genes of globally common strains, including those of the most common strain, P[8]G1 [21–24]. Furthermore, numerous studies have detected novel human rotavirus G and P types that are not typeable because genotype-specific primers are absent from the multiplex primer mixtures [16, 25–28]. Finally, RT-PCR assays infrequently misclassify strains because of cross-priming of one genotype-specific primer on the nucleic acid molecule of another genotype. This phenomenon has been documented in studies from Hungary [22, 23] and Malawi [23]. To try to overcome these issues, new sets of primers or individual primers within a primer set have been designed by research groups involved in rotavirus strain surveillance [6, 22–24, 29]

Two new vaccines from Merck (RotaTeq) and GlaxoSmithKline (Rotarix) have at present been licensed in >40 and >90 countries respectively, and are being introduced into routine immunization programs in the United States, Europe, Latin America, and several other countries [30]. RotaTeq is a live, oral, pentavalent human-bovine reassortant rotavirus vaccine that contains a mixture of monoreassortments carrying the VP7 genes of human G1, G2, G3, and G4 and VP4 gene of human P[8] rotaviruses in the background of the genome of bovine WC3 rotavirus. The reassortants are expected to stimulate serotype-specific protection to these common rotavirus serotypes. In contrast, Rotarix vaccine is a monovalent, live attenuated human strain of the most common serotype, P1A[8]G1 and has been shown to elicit both homotypic and heterotypic immune responses and to cross-protect against different serotypes [31]. For countries anticipating the introduction of one or both of these new vaccines into routine immunization programs, it is vital to establish surveillance to assess their impact on serotype prevalence, monitor for the possible emergence of serotypes that escape vaccine induced immunity, and investigate the potential of both vaccines to undergo transmission in population or produce reassortants that cause rotavirus gastroenteritis.

The objectives of the present study were to provide a more complete picture of rotavirus strain distribution and epidemiology for Africa in anticipation of rotavirus vaccine introduction, by determining the G and P genotypes of previously nontypeable strains, and also to investigate why many of these strains from a study published elsewhere [32] were nontypeable with primer routinely used in the laboratory [8].

MATERIALS AND METHODS

Rotavirus-positive specimens. As described elsewhere [32], >3000 rotavirus-positive samples, collected from children <5 years of age in several African countries from 1996–2000, were analyzed in 3 African rotavirus workshops, organized between 1998 and 2000. Almost 75% of the strains were typed by VP7 MAb-EIA methods [5, 20, 33] or RT-PCR [8], but only half of the strains were subjected to VP4 genotyping by RT-PCR [7, 34]. For this present study, a subset of strains ($n = 90$) that could not be assigned both G and P genotypes were selected for further analysis. The analysis included an additional set of strains ($n = 125$), collected from 2002 to 2004, which also could not be assigned any G and P genotypes using the same approach just described (Table 1).

Genotyping of rotavirus strains. Rotavirus RNA was extracted from 10% (wt/vol) stool suspensions in phosphate-buffered saline using a NucliSens automated extractor (bioMérieux), according to the protocol specified by the man-

Table 1. Sample Origin and Year of Collection

Country	No. of samples, by year					
	1996–1998	1999	2000	2002	2004	Total
Nigeria	NA	10	5	8	NA	23
Cote d'Ivoire	NA	NA	9	19	7	35
Cameroon	5	NA	30	NA	NA	35
Zimbabwe	NA	NA	NA	2	NA	2
Burkina Faso	NA	3	NA	NA	NA	3
Sudan	NA	NA	NA	NA	1	1
Ethiopia	NA	NA	NA	NA	8	8
Botswana	NA	NA	NA	13	3	16
Ghana	NA	NA	NA	1	NA	1
Tunisia	NA	2	8	15	10	35
Kenya	8	10	NA	18	20	56
Total	13	25	52	76	49	215

NOTE. NA, no sample available.

ufacturer. RT-PCR was used to analyze G and P genotypes on the basis of previously determined conditions [6–8]. For G genotyping, a new set of primers were used for nested multiplex PCR; they included plus-sense primer 9con1-L (modified from 9con1) and type-specific primers (9T1–1, 9T1–2, 9T-3P, 9T-4, and 9T-9B) specific for G1–G4 and G9 [6]. In addition, minus-sense primer 9T1-Dg specific for G1 was also added to the G primer pool. For P genotyping, generic primers con3/con2 [7] were used to produce 876-bp fragments of VP8* of the VP4 gene. The 1T-1 to 5T-1 type-specific primers [7] were used against the con3 primer in all nested multiplex PCR analyses. Two additional minus-sense P[8]-specific primers (Jrg237, nucleotides 339–356, 5′ CGT GCA ATT GGG TCA TCT 3′ [35]; 1T1-V, nucleotides 339–356, 5′ CGT GCA GCT AGG TCA TCT 3′), based on the sequence of genetically distinct P[8] strains, were included in the pool of P genotyping primers (unpublished data).

Strains that could not be genotyped by these modified methods were identified by nucleotide sequencing. For sequence analysis, a 896-bp length of the VP7 gene and a 876-bp fragment of the VP8* region of VP4 gene of nontypeable samples, were generated with the same consensus primers used for seminested genotyping RT-PCR [6, 7], using a one-step RT-PCR kit (Qiagen) according to the manufacturer's instructions. After denaturation of the double-stranded RNA, as described elsewhere [36], RT was carried out for 30 min at 50°C, followed by 15 min at 95°C to inactivate the reverse transcriptase and activate the DNA polymerase.

The complementary DNA was then subjected to 30 cycles of PCR at the following conditions: 30 s at 94°C, 30 s at 50°C, and 45 s at 72°C, followed by a 7-min extension at 72°C and a 4°C cooling step. Amplicons were run in a 1% agarose gel, and the desired band was cut out and purified with the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's protocol. Cycle sequencing of each amplicon was then conducted with same consensus primers described above, using the BigDye Terminator cycle sequencing kit (Applied Biosystems). Cycle sequencing products were purified using CentriSep spin columns (Princeton Separations), dried in a DNA SpeedVac (Savant Instruments), and reconstituted in 15 μL of Hi-Di formamide.

Automated separation and base calling of cycle sequencing products was performed using an ABI 3130 sequencer (Applied Biosystems). Sequences were aligned with the Sequencher program (version 4.8; Gene Codes) and subsequently compared with other sequences by using the University of Wisconsin Genetics Computer Group suite of sequencing software (version 11.1) [37]. All mixed infections identified during genotyping PCR were confirmed by employing VP4- and VP7-specific probes and methods described elsewhere [38]. An additional G1-specific probe (G1-P3, nucleotides 152–175; 5′-ATA GAT CTT TGT TGA TTT ATG TAG-3′) based on the sequence of a variant G1 strain A91A from GenBank (accession no. M93006), was used for confirmation.

Nucleotide sequence accession numbers. The nucleotide sequences of the VP7 genes of 17 rotavirus isolates have been submitted to the GenBank database and assigned accession numbers for the G10 isolates (6717/2002/ARN, EF218662; 6721/2000/ARN, EF218663; 6730/1999/ARN, EF218664; 6748/2002/ARN, EF218665; 6755/2002/ARN, EF218666); G5 isolates (6784/2000/ARN, EF218667); and G8 isolates (6780/2000/ARN, EF218668; 6782/2000/ARN, EF218669; 6785/2000/ARN, EF218670; 6786/2000/ARN, EF218671; 6787/2000/ARN, EF218672; 6804/2000/ARN, EF218673; 6809/2000/ARN, EF218674; 6810/2004/ARN, EF218675; 6854/2002/ARN, EF218676; 6862/2000/ARN, EF218677; 6736/2004/ARN; EF218678).

RESULTS AND DISCUSSION

Using these modified RT-PCR procedures we were able to identify 190 of 215 (88.4%) G genotypes and 193 of 215 (89.8%) P genotypes. Twenty-five (11.6%) and 22 (10.2%) samples could not be assigned a G or P genotype, respectively, although a first-round RT-PCR product was obtained for each using consensus primers. By nucleotide sequencing and comparison with previously published sequences in the GenBank database, these samples were shown to belong to types G10 (5 of 25; 20%), G8 (11 of 25; 44%), G5 (1 of 25; 4%), G9 (2 of 25; 8%), and G1 (6 of 25; 24%). Among 22 P untypeable samples sequenced, 12 genotype P[8] (54.5%) and 10 genotype P[6] (45.5%) strains were identified by comparison with related strains in the database.

A total of 13 single G- and P-type combinations, consisting of 7 different G types and 3 different P types, were identified (Table 2). This subset of samples showed more variability than

Table 2. P and G Genotypes Found by Reverse-Transcription Polymerase Chain Reaction, Hybridization, and Sequence Reaction in 215 Untypeable Rotavirus-Positive Samples from the African Rotavirus Network (1996–2004)

	No. (%) of samples, by G genotype								
P genotype	G1	G2	G4	G5[a]	G8[a]	G9	G10[a]	Mixed[b]	Total
P[4]	0	1	0	0	0	0	0	0	1 (0.5)
P[6]	3	10	0	0	2	3	1	5	24 (11.2)
P[8]	101	6	2	0	5	16	4	39	173 (80.5)
P[7]	0	0	0	1	0	0	0	0	1 (0.5)
P[6]P[8]	6	4	0	0	4	0	0	2	16 (7.4)
Total (%)	110 (51)	21 (10)	2 (0.9)	1 (0.5)	11 (5.1)	19 (9)	5 (2.3)	46 (21.4)	215 (100)

[a] Detection was done by sequencing.
[b] Includes 16 samples with G1 plus G2, 14 with G1 plus G9, 2 with G4 plus G9, 2 with G2 plus G9, 5 with G1 plus G4, 2 with G4 plus G3, 2 with G4 plus G2, 1 with G4 plus G1G2, and 2 with G3 plus G4G1.

seen in recent full studies from the African continent, where 7–9 different single G-P combinations were observed [32, 36]. As expected, strain P[8]G1 (46.9%) was predominant among nontypeable samples. As in other studies [23, 24], the failure to identify P and G genotypes for many of these strains by multiplex RT-PCR could be explained by variation in the primer binding sites of P[8]-, P[6]-, and G1-specific primers designed in the early 1990s and based on very few strains [7, 8]. For G1 strains, the nucleotide sequence alignment showed 1–3 mutations further away from both the 5′ and 3′ ends of the primer binding sites (data not shown). Moreover, the 6 G1 strains that could not typed with the Das G1-specific primer showed 2-point mutations in the middle of the primer binding site. For P[8] and P[6] strains, 3–5 mutations were detected at the primer binding sites of the specific primers 1T-1 and 3T-1, respectively (data not shown). However, with the addition of 2 newly designed P[8]-specific primers (JRG237 and 1T1-V), >85% of the strains were typed. These mutations at the primer binding sites of the G1-, P[6]-, and P[8]-specific primers decrease the affinity of primer binding and may explain the failure to identify these strains. Failed genotyping or mistyping and the use of multiple primer sets or newly designed primer pairs specific for local strains have already been reported from different parts of the world [21–24, 39, 40]. Because of natural variation in rotaviral gene sequences, close monitoring of rotavirus genotyping methods are extremely important.

Mixed infections were also detected in 46 of 215 (21.4%) G genotypes and 16 of 215 (7.5%) P genotypes. The mixed G genotypes observed were G1G9 (14 of 215; 6.5%), G1G2 (16 of 215; 7.4%), G4G1 (5 of 215; 2.3%), 2 samples each had G4G3G1, G4G9, G2G9, G4G3, G4G2 infections and 1 sample had G4G2G1 infection. All mixed P genotypes were P[6]P[8]. Southern hybridization assay with VP4 and VP7 genotype-specific probes was used to confirm that all the mixed G and P types were true mixed infections (data not shown). The detection rate of mixed infections in the random selection of nontypeable samples (215 of ~750) reported in this study is substantially lower than what was reported in Guinea-Bissau [10, 39]. That study involved a collection of previously incompletely typed rotavirus strains, and mixed G and P types were detected in 59% and 33% of strains, respectively. High frequencies of mixed infections have been reported in nonselective studies in various countries, including India [41, 42], Bangladesh [43], Vietnam [44], and Brazil [38, 45]. Frequent detection of mixed infections in diarrheic children may play a significant role in generating strain diversity.

Several unusual rotavirus genotypes including G10, G8, and G5 were detected. These strains are commonly found in animals, particularly cattle and pigs. However, during the past decade, many reports have documented detection of human rotavirus strains in close relationship with animal rotaviruses [46] and high regional diversity among circulating strains [23, 32]. The G10, G8, and G5 strains detected in this study showed strong relationships to those reported elsewhere or submitted to the GenBank database. The genotype G10 strains exhibited the highest similarity (≥99%) to the nucleotide and amino acid sequences of human rotavirus strains 1784CI (accession no. AY816181) and 3008CM (accession no. AY816182), previously isolated in Africa and elsewhere, strongly suggesting that they belong to serotype G10. The G8 strains shared nucleotide and amino acid homologies of ≥97% relative to 2 human strains, SI-885 (accession no. DQ995179) and HMG89 (accession no. X98918), isolated from Slovenia and Nigeria respectively, indicating that they belong to serotype G8. The G5 strain, the second isolated from the African continent and from the same location, shared nucleotide (100%) and amino acid (99%) homologies with human G5 strain MRC3105 (accession no. AY327107) reported in Cameroon [47], which is more distantly related to porcine G5 strain OSU (accession no. X04613; 86% nucleotide and 94% amino acid homologies). A more detailed analysis of these unusual strains will appear later (unpublished data).

Although the modified methods used here allowed us to type ~90% of a collection of strains that were 100% untypeable with the originally described genotyping procedures, we still found about 10%–12% of the strains that could not be genotyped with the modified methods. All of the P genotyping failures were due to genetic variation in the primer binding site of the globally most common genotype P[8] strains. Our findings reinforce the need to develop alternative or modified methods to genotype rotavirus strains more reliably, and also highlight the value of using >1 set of genotype-specific primers [10].

The findings reported in this investigation have helped give a more complete picture of the strains circulating among the African Rotavirus Network participating countries. Furthermore, the detection of genotypes G10, G8, and G5 reinforces findings that these strains are globally distributed and strengthens the need to continue strain surveillance. Because each of these strains belonged to genotype P[8], it will be interesting to investigate whether they represent multiple-gene reassortants or whether only the VP7 gene reassorted into a genetic background of typical long electropherotype P[8] strains of the Wa genogroup during coinfections in vivo.

THE AFRICAN ROTAVIRUS NETWORK

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