Molecular Characterization of Rotavirus Strains Circulating among Children with Acute Gastroenteritis in Madagascar during 2004–2005

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A survey was undertaken of the etiology of acute gastroenteritis in children <16 years of age in Antananarivo, Madagascar, from May 2004 through May 2005. With use of electron microscopy of fecal specimens, 104 (36%) of 285 children were found to be infected with rotavirus. Rotavirus strain characterization was undertaken using enzyme-linked immunosorbent assay, electropherotyping, reverse-transcription polymerase chain reaction genotyping, and nucleotide sequencing. The predominant group A rotavirus strain types identified were P[4]G2 (62%) and P[8]G9 (23%). Nucleotide sequence analysis of the VP7 genes of selected Malagasy G2 and G9 strains demonstrated similarity with those of other recently identified African rotavirus strains belonging to the same genotype.

Diarrheal disease is a leading cause of morbidity and mortality among children and is responsible for 2.5 million childhood deaths each year in developing countries [1]. Rotavirus is the major cause of acute dehydrating diarrhea in children <5 years of age worldwide and is estimated to be responsible for over half a million childhood deaths annually [2]. The majority of these deaths occur in developing countries in Africa and Asia [2].

Human rotavirus is an icosahedral virus of 75 nm in diameter with a triple-layered capsid and no envelope [3]. Its genome comprises 11 segments of double-stranded RNA. Epitopes on the middle layer, which consists of virus protein (VP) 6, determine rotavirus group and subgroup. Most rotavirus strains that infect humans are in group A. Two other proteins, VP4 (encoded by genome segment 4) and VP7 (encoded by segments 7, 8, or 9, depending on the strain), make up the outer layer. These 2 proteins independently induce neutralizing antibodies. VP4 determines the P (or protease sensitive) type, whereas VP7 is a glycoprotein and confers G type specificity. Most rotavirus strains infecting humans are of G1, G2, G3, G4, and G9 type and of P[8], P[6], and P[4] type [4, 5]. However, other G types are important in some settings (eg, G5 in Brazil [6], G8 in Malawi [7], and very recently, serotype G12 has emerged globally [8]). Two live, attenuated, oral rotavirus vaccines have been developed that have the potential to decrease the global burden of rotavirus disease [9]: Rotarix (GlaxoSmithKline Biologicals), which is a monovalent P[8]G1 attenuated human rotavirus strain, and RotaTeq (Merck), which is a pentavalent vaccine consisting of a mixture of 5 human-bovine monoreassortants expressing G1–G4 and P[8].

Madagascar is a large island nation in the Indian Ocean off the coast of southeastern Africa. It is the fourth largest island in the world and is home to 5% of the world's plant and animal species (80% of which are indigenous to Madagascar). It has a population of ~18.6 million persons, with an annual growth rate of

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2.6% but a mortality rate of 85,000 deaths per year among children <5 years of age. There are very few data describing rotavirus infection in Madagascar, with the most recent publication dating from 1993 [10]; although information was presented on rotavirus electropherotypes [10], there are no data available on rotavirus G and P types in Madagascar. Thus, this study was undertaken to investigate the relative frequency of rotavirus genotypes in Antananarivo, Madagascar, and to examine how the VP7 genes of the predominant genotypes were related to the corresponding genes of rotavirus isolates from continental Africa.

MATERIALS AND METHODS

Stool samples were collected from children <16 years of age who presented with acute diarrhea to hospitals and rehydration clinics in Antananarivo, Madagascar. Antananarivo is the capital and largest city in Madagascar, with a population of ~1.5 million. It has a temperate climate, with 3 seasons: cold (April to October), wet (October to December), and dry and warm (December to April). Participating hospitals and health facilities included the university hospital pediatric unit, a government pediatric hospital, a military hospital (pediatric and emergency units), dispensaries, and the Institut Pasteur de Madagascar. The collection period was from May 2004 through May 2005. Fecal samples were frozen at −80°C and transported to the University of Liverpool, United Kingdom, for further investigation.

All specimens were first screened by negative stain electron microscopy. Specimens containing rotavirus were then further examined by enzyme-linked immunosorbent assay (ELISA; Rotaclone; Meridian Diagnostics), according to the manufacturer's instructions, to confirm the presence of group A rotavirus antigen. Fecal samples containing group A rotavirus were then characterized for genomic RNA migration patterns (electropherotype) and G and P genotypes. In brief, a 10% v/v fecal suspension was made in phosphate-buffered saline, and RNA was extracted using a guanidine isothiocyanate–silica method [11]. The RNA was eluted using 70 μL of RNAse-free water and used directly for polyacrylamide gel electrophoresis (PAGE), as described elsewhere [12], and for G and P typing. Rotavirus G and P typing was undertaken for all group A rotavirus strains identified with use of a heminested multiplex reverse transcription-polymerase chain reaction (RT-PCR) method with primers to detect genotypes G1–4, G8, G9, G12, P[4], [6], P[8], P[10], and P[11] [11, 13–15]. Type-specific RT-PCR products were resolved by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and visualized by UV transillumination.

To examine the VP7 sequence diversity among the major G types identified, the 9con1/9con2 amplicons of selected strains were subjected to nucleotide sequencing. All RT-PCR amplification products were purified using Micro-spin columns (GE Healthcare) and were sequenced using Cogenics (Hope End). Phylogenetic trees were constructed according to the neighbor-joining method [16] in the CLUSTAL W software package [17].

RESULTS

A total of 285 fecal samples were obtained from children with acute dehydrating diarrhea for whom the median age was 20 months (range, 1 day to 16 years). Among the 285 specimens examined by electron microscopy, 104 (36%) contained rotavirus. All 104 specimens were positive for group A rotavirus antigen by ELISA. The median age of the rotavirus-infected children was 10 months (range, 1 day to 48 months). The detection rate of rotavirus was highest (>50%) during the drier and cooler months of April, May, and June (data not shown).

Among the 104 group A rotavirus strains characterized by RT-PCR, the genotypes identified were P[4]G2 (62%), P[8]G9 (23%), P[8]G1 (6%), and P[6]G1 (1%). Two strains (2%) contained >1 G or P type. Seven strains (7%) could not be fully characterized for either G or P type (6 strains) or for both G and P type (1 strain) (Table 1).

Rotavirus-containing specimens with sufficient amount of stool ($n = 102$) were analyzed for genomic RNA patterns with use of PAGE. Forty-one short electropherotypes were identified that belonged to genotype P[4]G2 ($n = 39$), P[NT]G2 ($n = 1$), and P[4]GNT ($n = 1$). Among 14 long electropherotypes identified, associated genotypes included P[8]G9 ($n = 9$), P[8]G1 ($n = 2$), P[6]G1 ($n = 1$), and P[8]G1/G9 ($n = 1$). For 30 specimens the resolution of genome segments 10 and 11 was not sufficiently clear to assign either a short or long RNA pattern because of an insufficient RNA concentration. In 17 specimens, no rotavirus-specific bands were detected using PAGE.

Sequence analysis demonstrated that the VP7 genes of G2 strains from Madagascar were similar to each other, sharing nucleotide sequence identities of >99.2% and amino acid identities of >99.3% (data not shown). Phylogenetic analysis confirmed that Madagascar G2 VP7 genes clustered together and were in a large branch consisting of recent African G2 VP7

Table 1. Rotavirus G and P Genotypes in Madagascar

	P type					
G type	P[4]	P[6]	P[8]	P[4]/P[8]	P[NT]	Total
G1	…	1	6	…	…	7
G1/G9	…	…	1	…	…	1
G2	64	…	…	1	2	67
G9	…	…	24	…	…	24
GNT	2	…	2	…	1	5
Total	66	1	33	1	3	104

NOTE. NT, nontypeable.

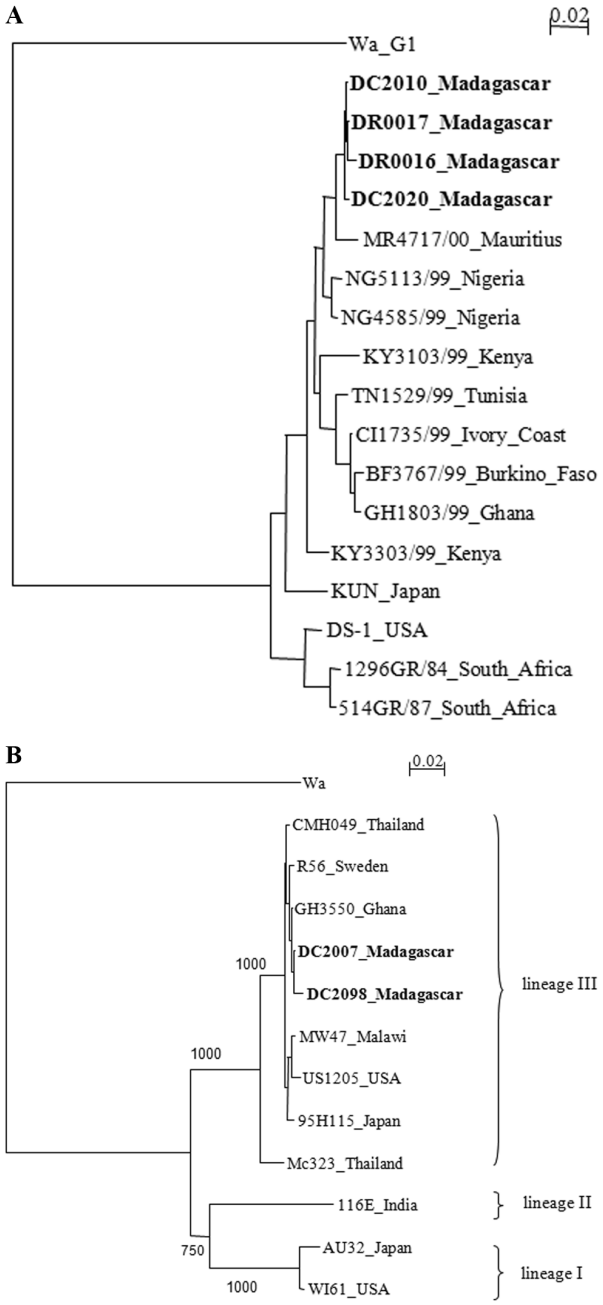

Figure 1. Phylogenetic trees based on VP7 nucleotide sequences of representative Madagascar G2 strains and selected G2 strains in the DNA databases *(A)* and similarly for selected G9 strains *(B).* The serotype G1 strain Wa is included in each tree as an outgroup. The strain designations are followed by country of origin. The strains from Madagascar are highlighted in bold. Horizontal lengths are proportional to the genetic distance calculated with Kimura's 2-parameter method. Scale bar shows genetic distance expressed as nucleotide substitutions per site. The number adjacent to the node represents the bootstrap value out of 1000 replicates; only probabilities of $\geqslant$750 per 1000 trials are indicated. Strain designation and nucleotide accession numbers (in brackets) for the field strains from Madagascar are as follows: *A,* DC2020 (FJ436809), DC2010 (FJ436810), DR0016 (FJ436811), and DR0017 (FJ436812); *B,* DC2007 (FJ436813) and DC2098 (FJ436814). Strain designation and nucleotide accession numbers (in brackets) for the reference strains are as follows: *A,* Wa (K02033), MR4717/00 (AY261358), NG5113/99 (AY261352), NG4585/99 (AY261351), KY3103/99 (AY261349), TN1529/99 (AY261357), CI1735/99 (AY261354), BF3767/99 (AY261355), GH1803/99 (AY261353), KY3303/99 (AY261350), KUN (D50124), DS-1 (AB118023), 1296GR/84 (AY261334), and 514GR/87 (AY261338); *B,* Wa (K02033), CMH049 (AY699293), R56 (AY253837), GH3550 (AY211067), MW47 (AJ250544), US1205 (AF060487), 95H115 (AB045373), Mc323 (D38053), 116E (L14072), AU32 (AB045372), and W161 (AB180969).

genes (Figure 1*A*). The VP7 sequences of 2 G9 strains from Madagascar were clustered together and belonged to lineage III that contained globally predominant G9 strains (Figure 1*B*). Thus, for both G2 and G9 VP7 genes, Madagascar strains were not particularly unique but were part of continental African strains representing each genotype.

DISCUSSION

In this 13-month study of childhood gastroenteritis, 104 children (36%) were infected with rotavirus. The rotavirus detection rate is similar to that (36%) reported in a study from the Majunga region of Madagascar [18]. It is, however, significantly higher than the detection rates (10%–15%) reported in studies from other parts of the island that enrolled similar patients [10, 19, 20]. The young age of children with rotavirus gastroenteritis in Madagascar (median age, 10 months) and higher rotavirus detection rates during drier, cooler months (April to June) are findings consistent with studies from elsewhere in sub-Saharan Africa [21].

To our knowledge, this is the first study to document the rotavirus G and P types causing diarrheal disease in Madagascar. The majority of rotavirus strains (85%) were composed of 2 strain types: P[4]G2 (62%) and P[8]G9 (23%). The globally most common P[8]G1 genotype comprised only 6% of strain types. In recent global reviews, serotypes G1, G3, G4, and G2 were the most important in decreasing order of prevalence, with rotavirus strains of P[4],G2 genotype representing only 11%–12% of typed strains overall [4, 5]. The distribution of rotavirus serotypes may vary markedly by country, however, with globally uncommon serotypes predominating in some settings [22]. In Africa, P[4]G2 strains accounted for 16% of the strain types before 1997 [21] and 5% of strain types in studies published during 1997–2006 [23]. Our current data from Madagascar are in marked contrast to a recent 10-year study from Malawi in which <1% of strains were genotype P[4]G2 [24]. Of note, in the 1993 study in Madagascar [10], 82% of strains were of short electropherotype, suggesting the possibility that P[4]G2, short electropherotype rotavirus strains may have been predominant at least 15 years ago. Nucleotide sequence analysis of G2 strains in the current collection from Madagascar showed close homology with G2 strains detected in South Africa [25]. Of note, the Madagascar G2 strains shared amino acid residues in antigenic regions of the VP7 protein at positions 87 (Threonine), 96 (Asparagine; region A), and 213 (Aspartic acid; region C) with those from South Africa, Nigeria, Ghana, Mauritius, and Kenya (data not shown). These amino acid substitutions were previously noted to be characteristic of African G2 strains, with the exception of a single Kenyan strain [25].

Serotype G9 has emerged to become an important global serotype, and Madagascar can now be added to the list of countries where it has been found in significant numbers. The VP7 gene sequence of 2 G9 strains showed close homology with other recently identified G9 strains, including a strain from Ghana. A single P[6]G1 strain was detected in the current study and was reported in 3% of rotavirus strains in a review of African studies [23]. Seven strains (7%) could not be characterized for G and/or P type; these may represent common strains that have failed to type (eg, because of insufficient RNA or mutations in the primer binding sites) or uncommon serotypes not targeted by the primers used in this study. Of note, we detected neither rotavirus strains of serotype G12, nor the G8 strains that are particularly prevalent in Africa [4, 5, 7]. The marked differences in rotavirus serotype distributions with those in neighboring countries highlight the importance of continued surveillance of rotavirus strain types both before and after rotavirus vaccine introduction in Madagascar.

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