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Geographic Distribution of Human Rotavirus VP4 Genotypes and VP7 Serotypes in Five South African Regions

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The rotavirus outer capsid proteins elicit the production of neutralizing antibodies and are known to play a role in inducing resistance to disease. In this study, cDNA probes directed at the six most common human rotavirus VP7 serotypes (G1 to G4, G8, and G9) and five human rotavirus VP4 genotypes (P4, P6, P8, P9, and P10) were utilized. Hybridization analysis of 572 human rotavirus strains collected from five regions in South Africa was performed to determine the distribution of the VP7 serotypes and VP4 genotypes in nature. VP7 serotype G1 was identified most frequently, occurring in 51% of the rotavirus strains tested. VP7 serotypes G2 and G4 occurred in similar numbers, although their distribution varied regionally. Few serotype G3 strains and no G8 or G9 strains were identified. The P8 VP4 genotype occurred most frequently overall (66%), and the P4 genotype was detected next most frequently. The P6 genotype was identified in 28 symptomatically infected neonates and in 8 symptomatic infants. Few P9 strains were identified. The potential for reassortment events was demonstrated by dual infections with different viruses.

In developing countries, rotavirus has consistently been identified as the most frequent cause of diarrheal illness in children under 5 years of age. In addition, it has been estimated that in a single year rotavirus infection may account for up to 1 million deaths (19). Development of an effective vaccine against rotavirus would diminish the high infant mortality associated with this disease.

The two outer capsid proteins of the virus, VP4 and VP7, both of which elicit the production of neutralizing antibodies, have been shown to play an important role in inducing resistance to the disease (17, 25). While several reports have described the relative frequency and distribution of VP4 and VP7 in various settings, no significant reports of the natural distribution of these viral markers have emerged from Africa.

VP7 is an outer capsid protein which is encoded by gene segment 7, 8, or 9 (depending on the strain) and which defines the rotavirus serotype (2, 36). The VP7 serotype numbering system utilizes the prefix G, for glycoprotein (5). Nine rotavirus VP7 serotypes have been identified in humans. Serotyping studies conducted in human populations have shown the epidemiological importance of VP7 serotypes G1, G2, G3, and G4 (3, 10, 24, 33, 35). Serotypes G8 and G9 have been identified far less frequently in humans (23, 35), and a G12 rotavirus serotype has also been reportedly recovered from humans (34). Recently, the classical bovine rotavirus serotypes, G6 and G10, have also been recovered from young children (4, 11).

In most studies to date, VP7 serotype G1 strains have been the most frequently detected serotype (3, 10, 21, 24, 33), although other VP7 serotypes may predominate at certain times in certain locations (35).

VP4, encoded by the fourth viral gene, also exhibits antigenic polymorphism (15, 17, 31). However, VP4 has proved difficult to classify serologically, and various genetic relatedness techniques, such as nucleic acid sequence and hybridization analyses, have been utilized more thoroughly (8, 13, 14, 20, 29). VP4 has thus been termed a "genotype," and six human VP4 genotypes have now been recognized. Recently, a unified numbering system was proposed for the VP4 genotype; it includes the prefix P, for protease sensitive (5).

The initial reports described two VP4 genotypes, P8 and P4 (Wa-like and DS-1-like gene alleles), which were recovered from children with symptomatic rotavirus infection. A third P6 genotype (M37-like allele) was recovered from neonates with asymptomatic infection in newborn nurseries (8, 13, 14, 18, 30). The VP4 P8 genotype has been detected in strains with VP7 serotype G1, G3, G4, and G9 specificity, while the P4 genotype is associated with rotaviruses bearing VP7 serotype G2 specificity (17, 29). The VP4 P6 genotype has been detected in rotaviruses bearing VP7 serotype G1, G2, G3, or G4 specificity (8, 13, 14, 18).

Occurring more rarely, three other VP4 genotypes have been described in human rotaviruses. Genotype P9, derived from strain K8, is associated with VP7 serotype G1 or G3 and was identified in Japan (32). The P10 genotype (strain 69M) was recovered from children with gastroenteritis in Indonesia (26). A sixth VP4 genotype, P12, has been described in children infected with a serotype G6 rotavirus (12).

In this study, cloned cDNA probes to the six most common VP7 serotypes (G1, G2, G3, G4, G8, and G9) and to the five most common VP4 genotypes (P4, P6, P8, P9, and P10) were used to determine the distribution of the VP4 genotypes and VP7 serotypes present in rotavirus field strains from five regions in South Africa.

MATERIALS AND METHODS

Rotavirus-positive specimens. Rotavirus field strains were collected in 1988 and 1989 from each of various locations in South Africa (Fig. 1) and were available in this laboratory for this study to investigate the distribution and diversity of VP4 genotypes and VP7 serotypes in human rotaviruses. Rotavirus-positive strains were obtained from Estelle Baxter, South African Institute for Medical Research, Port Elizabeth (n = 130); Fiona Griffiths, University of Transkei, Umtata (n = 71); Errol Gove, Niehaus and Botha Pathology Laboratories, Pretoria (n = 130); and Sarah Wanda, van Drimmelin Pathology Laboratories, Johannesburg (n = 118). Specimens were also available from our own laboratory in Ga-Rankuwa (n = 123).

Extracted double-stranded RNA from either stool- or tissue culture-adapted material of the field strains was prepared by phenol-chloroform extraction and ethanol precipitation.

PCR-generated probes. PCR was used to generate DNA probes from each of

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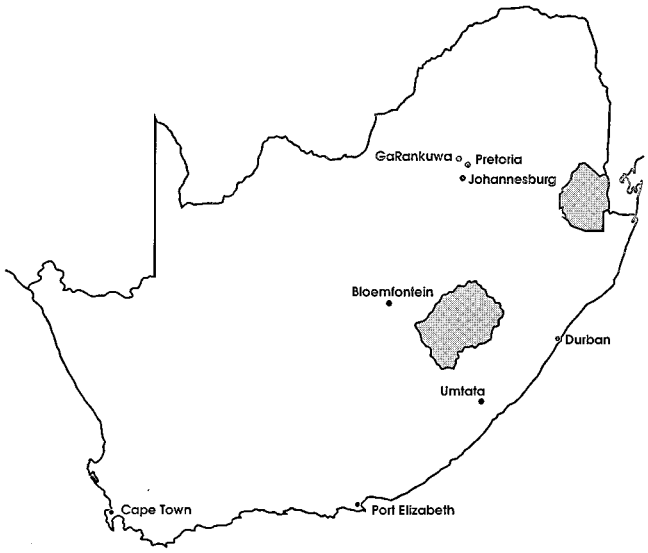

FIG. 1. Diagrammatic map of South Africa showing the areas from which the rotavirus strains were collected for this study.

the VP4 genotype and VP7 serotype clones, as described elsewhere in detail (7, 20, 28). The PCR-generated probes were retrieved by electrophoresis through 1.4% low-melting-point agarose gels and recovered by the method of Feinberg and Vogelstein (6). The probes were radiolabelled with [^{32}P]dATP, using a random primer labelling kit (Prime-a-gene; Promega) as specified by the manufacturer.

Hybridization analysis. The viral double-stranded RNA was denatured by boiling for 3 to 5 min and then immediately chilled on ice for 2 to 5 min. Several identical nylon membranes (Nytran; Schleicher & Schuell) were prepared by dotting 2 μl of the RNAs onto the membranes and then exposing the membranes to short-wave UV light for 5 min. Control RNAs from the prototype laboratory strains with known VP4 and VP7 specificity were similarly dotted on separate strips for inclusion in each hybridization. These included strains Wa (G1 P8), DS-1 (G2 P4), P (G3 P8), ST3 (G4 P6), 69M (G8 P10), Wi61 (G9 P8), and AU228 (G3 P9).

Hybridization was performed as described previously (7, 28). Briefly, the membranes were prehybridized for 1 to 2 h at 52 or 54°C in a mixture containing 50% formamide, 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 40 mM sodium buffer (pH 6.5), 0.2% sodium dodecyl sulfate (SDS), and 2× Denhardt's solution. A second prehybridization step was conducted in fresh solution containing 25 μg of boiled salmon sperm DNA per ml. Hybridization was carried out in a similar solution containing 10% dextran sulfate and 3×10^6 to 5×10^6 cpm of the appropriate probe. Hybridization was carried out at 54°C (VP4 probes) or 52°C (VP7 probes) for 12 to 24 h and was terminated by washing the membranes four to five times in 2.5× SSC with 0.2% SDS at room temperature and twice in 1× SSC with 0.1% SDS at the hybridization temperature. The membranes were dried at 37°C and exposed to autoradiographs for 12 to 24 h.

RESULTS

Specificity and sensitivity of the probes. The sensitivity and specificity of the VP7 and the VP4 probes used in this study have been previously established (7, 20, 28). Altogether, 572 specimens from five different locations in South Africa were evaluated with the cloned VP7 and VP4 probes to assess the distribution of the different VP7 serotypes and VP4 genotypes in nature (Tables 1 and 2).

Distribution of VP7 serotypes. A total of 418 specimens (73.1%) could be typed by the VP7-specific probes. The majority were typed as G1 (51%), with virtually equal numbers of G2 and G4 strains (21.8 and 23.4%, respectively). Only nine specimens were typed as G3 strains (Table 1). No serotype G8 or G9 strains were detected. In six specimens, dual reactivity was noted with more than one of the probes (G1 and G2 [n = 3], G1 and G3 [n = 1], G1 and G4 [n = 1], and G2 and G4 [n = 1]), which indicated a mixed infection with more than one viral strain.

TABLE 1. Distribution of VP7 serotypes in South African field strains of rotavirus evaluated with PCR-generated probes

Region	No. of strains tested	No. with given serotype						
		G1	G2	G3	G4	G8	G9	Mix
Pretoria	130	67	13	3	20			2
Ga-Rankuwa	123	40	16		44			2
Johannesburg	118	36	10	4	30			
Umtata	71	41	14					1
Port Elizabeth	130	30	38	2	4			1
Total	572	214	91	9	98	0	0	6

Geographically, the G1 strains were predominant in all regions, except Port Elizabeth, where G2 strains were the most common (Table 1). Although G1 and G2 strains were present in all regions, it was of interest to observe that serotype G4 strains were common in the Pretoria–Ga-Rankuwa–Johannesburg region (Fig. 1) and seemed to be concentrated in this area of the country during the study.

Distribution of VP4 genotypes. In addition, 383 specimens (67.0%) were typed by the VP4 genotype-specific probes. Of the specimens which were typed, the P8 genotype was observed most frequently in all geographical areas examined, except Port Elizabeth, where the P4 genotype occurred slightly more commonly (Table 2). Overall, 66% of the strains carried the P8 genotype. The P4 genotype was identified next most commonly in 83 cases (21.7%).

The P6 VP4 genotype was observed in 37 cases (9.7%) of those specimens typed (Table 2). In all but one case from Pretoria, the P6 genotype was identified from neonates symptomatically infected with rotavirus. At Ga-Rankuwa, 10 of the P6-bearing strains were recovered from neonates with symptomatic rotavirus infection, while the remaining four cases and the four cases from Port Elizabeth were all observed in infants and young children with symptomatic rotavirus infection.

Strains bearing the P9 VP4 genotype were identified in five specimens (1.3%), indicating that, although rare, the distribution of this VP4 genotype is more widespread than previously thought. The presence of the P9 gene in these strains was confirmed by Northern (RNA) blot hybridization (data not shown).

Of interest, though, is that 23 of the field strains could not be typed by the probes used in this study, although they reacted with a common VP4 probe, indicating the presence of an untypeable VP4 gene in South Africa.

TABLE 2. Distribution of VP4 genotypes in South African field strains of rotavirus evaluated with PCR-generated probes

Region	No. of strains tested	No. with given genotype						
		P8	P4	P6	P9	P10	VP4[a]	Mix
Pretoria	130	65	13	19	2		1	2
Ga-Rankuwa	123	44	12	14	2		20	2
Johannesburg	118	72	10				2	
Umtata	71	40	14		1			1
Port Elizabeth	130	32	34	4				
Total	572	253	83	37	5	0	23	5

[a] A VP4 common probe provided by Jorge Flores, National Institute of Allergy and Infectious Diseases.

TABLE 3. Combinations of VP7 and VP4 identified in single rotavirus strains

VP4 genotype	VP7 serotype (no. of strains)			
	G1	G2	G3	G4
P8	212		1	40
P4		83		
P6	1			36
P9	1		3	

Five of the six strains which reacted with more than one VP7 probe also reacted equally well with more than one of the VP4 probes used in this study (P8 and P4 [$n = 3$], P8 and P9 [$n = 1$], and P8 and P4 [$n = 1$]).

Combinations of VP4 and VP7 identified. Table 3 shows the correlation of the VP4 genotype with the VP7 serotype identified in the same specimen. In every case of a VP7 G2 serotype strain, the P4 VP4 genotype was identified. The P8 genotype was found to occur predominantly with serotype G1 strains, although an association occurred with both a G3 strain and several G4 strains (Table 3).

The P6 genotype was found to occur predominantly in serotype G4 strains, which may be related to the regional distribution of this genotype. Serotype G4 strains were circulating in the neonatal nurseries in Pretoria and Ga-Rankuwa, where the majority of the P6 genotypes were identified. In addition, 20 strains with a serotype G4 specificity could not be typed by the VP4 probes used in this study.

The P9 genotype was identified in a few strains which carried a G1 or a G3 serotype specificity.

DISCUSSION

In this study, an assessment of the distribution of the different VP7 serotypes and VP4 genotypes of rotavirus field specimens collected from five locations in South Africa during 1988 and 1989 was made, using previously described PCR-generated probes. As was to be expected from previous studies conducted on the epidemiology of the VP7 serotype, G1 strains were identified most commonly overall (3, 10, 21, 23, 24, 33, 35). However, other VP7 types (G2 in Port Elizabeth and G4 in Ga-Rankuwa) were found to be predominant in localized regions during the course of the study.

Similarly, the P8 VP4 genotype was observed to occur most frequently among the rotavirus strains recovered from South African children. This epidemiological picture is similar to that reported previously (28, 29). It was interesting to observe that in Port Elizabeth the P4 genotype, the second most frequently detected VP4 (26% of those typed), was the predominant genotype. This was found to correlate well with the predominance of serotype G2 strains detected in Port Elizabeth during this study period.

These findings support the concept of the rotavirus "genogroup" (22) and demonstrate the strong presence of gene combinations in particular viral strains. For instance, members of the putative DS-1 genogroup (i.e., with a short RNA electrophoretype and a subgroup I VP6) were shown to carry the DS-1-specific G2 VP7 serotype and P4 VP4 genotype in all cases. In addition, the particular combination G4-P6 was demonstrated in viral strains from the Pretoria–Ga-Rankuwa region but not in those from elsewhere.

The diversity of rotavirus VP7 serotypes and VP4 genotypes in a specific geographical area indicates the need for continual monitoring of the circulating rotavirus strains in a specific community. This has been suggested previously for the VP7 serotype (35) and has important implications for future vaccine strategies.

The P6 genotype (with the M37-like VP4 allele) was detected in 37 specimens (9.7%). Previously, the P6 genotype has been reported to occur only in strains of the virus recovered from asymptomatically infected neonates (8, 13, 18, 30). In this study, 28 of the strains carrying the P6 genotype (18 from Pretoria and 10 from Ga-Rankuwa) were recovered from neonates symptomatically infected with rotavirus. Previous studies in this laboratory have demonstrated that a G4 serotype strain is circulating in the neonatal nurseries in Pretoria and Ga-Rankuwa (unpublished data).

The remaining nine P6 genotype strains were all observed in older infants with symptomatic rotavirus infection (four each from Port Elizabeth and Ga-Rankuwa and one from Pretoria). In all but one case, no other etiological agent which could be associated with the illness was identified. These results indicate that viral factors alone (i.e., the P6 VP4 gene) are unlikely to account for the attenuated infection in neonates.

Strains carrying the P9 VP4 genotype were identified in five patients (1.3%), indicating the global distribution of this VP4 genotype. The original P9-bearing strain was detected in Japan (32), and strains carrying the P9 genotype have been identified in Europe and Latin America, albeit at low frequency (28). As neutralization of the VP4 protein appears to be important for protection from illness (9, 16, 25), this finding may have some bearing on the implementation of future rotavirus vaccine strategies.

Six specimens reacted with more than one probe and were found on further examination to contain more than one strain of virus. The most common dual infection was found to occur between strains bearing a P8 and a P4 VP4 genotype (four specimens). Three of these specimens were shown to react with the serotype G1 and G2 VP7 probes, while one reacted with the serotype G4 and G2 probes. Dual infections between serotype G1 and G2 strains have been reported previously (on the basis of analysis of the VP7 serotype or RNA electrophoresis) and may be a result of the predominance of G1 and G2 strains in nature (1, 10, 24, 27, 28). In addition, a dual infection with a specimen bearing the P8 and P9 VP4 genotypes was also observed and the specimen reacted with the G1 and G3 VP7 serotype-specific probes. Both types of dual infection observed in this study occurred between members of the different human rotavirus genogroups (22), which indicates the potential for reassortant events between viruses from these genogroups.

Human rotavirus strains which could not be typed with the VP4 or the VP7 probes used in this study were identified in South Africa. Many of these specimens did not contain enough nucleic acid to react with the probes (119 specimens), as has been reported before (7, 20). Alternatively, some of these "untyped" strains may carry VP4 or VP7 genes which were not included in this study. For instance, the newly described human VP4 gene (PA169) (12) was not included in our analysis. In addition, although we included probes to the common human VP7 genes, recent studies have shown the presence of rotaviruses in human infants with the bovine VP7 serotype 6 or 10 genes (4, 11).

The development of these probes has enabled the examination of field isolates of rotavirus to determine the distribution of both outer capsid proteins in rotaviruses circulating in nature. This has revealed important epidemiological information on the distribution of rotavirus strains in the field. First, the overwhelming predominance of the G1 P8 strains (Wa genogroup) has been shown. Second, the regional (and temporal) differences in the distribution of circulating rotavirus strains

indicates the need for continual monitoring. Finally, the potential for reassortment to occur between viral strains with different VP7 and VP4 gene segments has been identified. This may have important implications for future vaccine development because reassortant viruses may arise in any region where a rotavirus vaccine is implemented.

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