SUPPLEMENT ARTICLE

Prevalence and Diversity of Rotavirus Strains in Children with Acute Diarrhea from Rural Communities in the Limpopo Province, South Africa, from 1998 to 2000

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Background. Data regarding the prevalence and molecular epidemiology of rotavirus infection in rural areas of Africa are limited. In this study the prevalence and genetic diversity of rotaviruses in a rural South African setting were investigated.

Methods. During June 1998 to June 2000, 420 stool specimens were collected from children with acute diarrhea who visited primary health care clinics in the rural Vhembe region, Limpopo Province, South Africa. Group A rotaviruses were detected by enzyme-linked immunosorbent assay, and the G and P types were determined by reverse-transcription polymerase chain reaction.

Results. Of the 420 specimens, 111 (26.4%) were positive for group A rotavirus; P[6]G1 strains predominated (32.4%), followed by P[8]G1 (13.5%), P[6]G9 (4.5%), P[4]G8 (3.6%), P[4]G1 (3.6%), P[6]G8 (3.6%), and P[6]G2 (2.7%). Dual infections, with >1 P type, were seen in 33 (37.1%) of the positive specimens.

Conclusion. The unusual serotype and genotype combinations of rotavirus circulating in the rural communities of the Limpopo Province highlight the need for more studies to monitor the geographic distribution of rotavirus strains in rural African settings.

Since their first description in 1973, numerous studies have confirmed the importance of human rotaviruses (HRVs) in severe dehydrating gastroenteritis in infants and young children, resulting in an estimated 440,000 deaths worldwide [1–5]. In sub-Saharan Africa it is estimated that HRVs causes ~145,000 deaths each year in children <5 years of age [6].

Rotavirus is a genus of the family *Reoviridae* and the viral particle is composed of 2 double-capsid layers that surround the viral core and enclose 11 segments of double stranded RNA [7]. Two structural proteins, namely the VP4 or P protein and the VP7 or G protein, form the basis for rotavirus classification. These proteins define the viral serotype and are the major antigens involved in virus neutralization and consequently play an important role in vaccine development [8]. The 4 most common VP7 serotypes, namely G1, G2, G3, and G4, in association with 2 VP4 genotypes, namely P[4] and P[8], were until recently considered to be the predominant serotypes causing pediatric diarrhea globally [9, 10]. Global epidemiological studies on rotavirus strains have identified P[8]G1, P[8]G3, P[8]G4, and P[4]G2 as the predominant circulating strains [11].

A number of studies have highlighted the presence of uncommon sero- and genotypes, and reassortments between common and uncommon strains, in children with gastroenteritis in different regions of the world [12–18]. In rural areas in developing countries, the lack

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of adequate sanitation and potable water supplies and the close association with domestic and farm animals, could give rise to reassortments between human and animal strains with the potential of cross-species infection [19]. The majority of rotavirus surveys in the Republic of South Africa (RSA) have been in urban and periurban areas, with stool specimens predominantly from hospitalized children [20–25]. A single study in a rural area in KwaZulu Natal, RSA, has shown that the predominant rotavirus strain was the P[8]G1 (22%) strain [26]. Very little information is therefore available regarding the HRV strains circulating in rural areas of RSA.

The Limpopo Province, in the northeast region of the RSA, covers an area of 123,910 square kilometers and has a population of ~5,500,000 people, of whom almost 603,000 are 0–4 years old [27]. The province has a subtropical climate with cool temperatures (5°C–27°C) during the winter season and mild temperatures (17°C–34°C) during the summer. The average annual rainfall in the summer season varies between 85 and 250 mm [28].

The socioeconomic status of the region is poor, public health services are minimal, and many communities have inadequately treated drinking water supplies. Only 78% of households have access to treated, partially treated, or untreated tap water, either via a tap in the dwelling or from a communal tap [29]. Approximately 89% of people in the region live in nonurban areas (average of 4.3 people per household), with almost 59.5% of these households still using wood fires for cooking [27]. Only 14.2 % of households in the province have rubbish removal once per week; 16.8% of households have access to flush or chemical toilets, but 46.7% households have pit latrines without a ventilation pipe [29]. Consequently a significant proportion (23.3%) of the population defecates in areas surrounding their dwellings and water sources [27]. These rural people keep cattle, donkeys, goats, pigs, chickens, cats, and dogs in their dwellings and the animals often use the same water source as the humans.

Rural communities in RSA are predominantly served by primary health care (PHC) clinics which are run by nurses, and many of these clinics serve between 1 and 3 villages with only about one-quarter of households being within walking distance of a clinic [27]. Diarrhea is treated symptomatically, with only serious cases being referred to the nearest hospital. No laboratory-based diagnoses are performed in these clinics.

The statistics for 1997–2001 indicated that intestinal infectious diseases were the leading cause of death among children aged 0–14 years in RSA [29]. Because the provision of safe drinking water and environmental interventions to improve hygienic and sanitation conditions may not necessarily reduce the prevalence of rotavirus diarrhea [30] and because there is no specific treatment for HRV diarrhea, vaccination will be the only effective means to control HRV-associated diarrheal disease [31]. Several different approaches to immunization against rotavirus have been pursued, but continued surveillance and identification of circulating strains globally remains crucial to determine the efficiency of these vaccines against uncommon circulating strains [32]. The aim of this study was to determine the prevalence of and to characterize HRV strains circulating among children <5 years of age living in impoverished rural areas in RSA.

MATERIALS AND METHODS

Informed consent and ethical approval. Verbal consent was obtained from the mother or the caretaker of the child before a stool specimen was obtained. Ethics committees of the Department of Health, Polokwane, Limpopo Province (Research approval, 8 June 1998), and the University of Venda, Thohoyandou, Limpopo Province, approved the study.

Specimen collection. From June 1998 to September 2000, diarrheal stool specimens ($n = 420$) were obtained from children <5 years of age who sought medical attention for acute gastroenteritis at primary health care clinics ($n = 10$) in the Vhembe region of Limpopo Province, RSA. Clinic workers recorded clinical symptoms and collected the stool specimens, which were kept at 4°C. The clinical data forms and stool specimens were collected from the clinics weekly. The undiluted stool specimens were transported on ice, reaching the laboratory within 5 h.

Detection of rotavirus antigens. A 10% suspension of each stool specimen was prepared in distilled water and stored at 4°C. Fecal suspensions were tested for group A HRV antigen using a commercial enzyme-linked immunosorbent assay kit (Premier Rotaclone EIA; Meridan Diagnostics), according to the manufacturer's instructions.

Polyacrylamide gel electrophoresis of genomic RNA. The electropherotypes of the isolates were determined through extraction of the viral RNA by phenol-chloroform treatment and ethanol precipitation [20], running the extracted RNA on vertical polyacrylamide slabs with 10% resolving and 3.5% stacking gels. The gels were loaded with 30 μL of extracted RNA and run at 100 V for 18 h at room temperature [33]. The RNA segments were visualized by silver staining [34].

Determination of VP6 subgroup. The VP6 subgroup specificity of the rotavirus isolates was determined by using a group-specific monoclonal antibody (MAb) enzyme-linked immunosorbent assay, with MAbs specific for subgroup I (clone 255/60) and subgroup II (clone 631/9) used as capture antibodies [35]. The MAbs used in this study were donated to the Medical Research Council Diarrhoeal Pathogens Research Unit, University of Limpopo, Medunsa Campus, where these assays were performed.

RNA extraction. Viral RNA was extracted from the 10% aqueous stool suspensions using TRIzol reagent (Invitrogen), according to the manufacturer's instructions, and resuspended

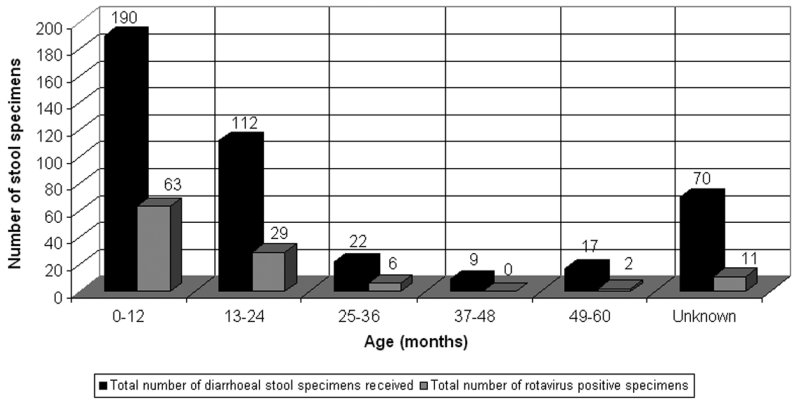

Figure 1. Number of diarrheal stool specimens that tested positive for rotavirus, according to patient age.

in a final volume of 15 μL of diethylpyrocarbonate (DEPC)–treated water (Invitrogen). For each extraction procedure, DEPC-treated water was included as a negative control.

P and G typing using reverse-transcription polymerase chain reaction. The VP4 gene was reversed transcribed and amplified with the Con2 and Con3 primers, followed by P typing using a cocktail of forward primers, namely Con3, 1T-1, 2T-1, 3T-1, 4T-1, and 5T-1, which are specific for the HRV P[8], P4, P6, P9, and P10 strains [36].

The VP7 gene was reverse transcribed and amplified with the sBeg9 and End9 primers, followed by G typing using the following cocktail of forward primers: End9, RVG9, aBT1, aCT2, aET3, aDT4, aAT8, and aFT9, which are specific for HRV G1, G2, G3, G4, G8 and G9 [37]. The G typing was confirmed by reverse-transcription polymerase chain reaction (RT-PCR) using the Das primers (G1–4, G8–9) and 9Con1 primers [38], specific for HRV G1, G2, G3, G4, G8, and G9 strains [31, 37, 38].

The PCR products were analyzed by gel electrophoresis in 2% SeaKem LE agarose gels (Cambrex BioScience) and visualized by ethidium bromide staining. Amplicons of the correct molecular weight were considered to indicate the presence of specific P and G types.

Sequence analysis. Two G9 strains belonging to VP6 subgroup I with short electropherotypes were selected for sequencing. The strains were cloned and sequenced to assess whether nucleotide mutations had taken place [39, 40]. Briefly, the VP7 gene amplicon was carefully excised from the 2% agarose gels and purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's specification. The purified product was cloned into the pGEM-T Easy Vector System (Promega) according to the manufacturer's instructions and the plasmid amplified in *Escherichia coli* JM101 cells. The cloned plasmid was extracted from the cells using the QIAprep Spin Miniprep Kit (Qiagen). The DNA was sequenced in both directions on an ALF Express automated sequencer using M13 primers [39, 40]. Nucleotide sequences from these specimens were compared, by pairwise analysis, with sequences from VP6 subgroup I rotaviruses from African human and animal sources with short electropherotypes [39, 40].

RESULTS

Detection of rotavirus antigen and clinical demographics. From June to December 1998, January to December 1999, and January to June 2000, 167, 183, and 70 specimens, respectively, were collected from children <5 years of age with diarrhea. These 420 stool specimens originated from the following clinics in the rural Vhembe region of the Limpopo Province: Makonde (20 specimens), Dumasi (31), Mutale Health Care (128), Phiphidi (75), Mukula (61), William Eddie (57), Sibassa (17), Pfananni (7), Tshiombe (13), and Vuwani (11). The majority (90%) of specimens were collected in the autumn and winter months, with peaks during June and July (results not shown). There were no data indicating what proportion of the diarrheal cases

Table 1. Clinical symptoms reported for rotavirus-positive and rotavirus-negative children with acute diarrhea

Symptom	No. (%) of patients	
	Rotavirus positive (n = 111)	Rotavirus negative (n = 309)
Diarrhea	111 (100)	309 (100)
Vomiting	75 (68)	92 (30)
Abdominal comfort	61 (55)	170 (55)
Fever	53 (48)	104 (34)
Nausea	53 (48)	56 (18)
Irritated	14 (13)	25 (8)
Listlessness	12 (11)	16 (5)
Respiratory symptoms	9 (8)	32 (10)

NOTE. Patients included children <5 years of age who presented with acute diarrhea at primary health care clinics in the Vhembe Region of Limpopo Province, South Africa.

Table 2. VP6 subgroup specificity and polyacrylamide gel electrophoresis (PAGE) RNA electropherotypes identified in 105 rotavirus-positive stool specimens

	No. (%) of specimens						
	By electropherotype		By VP6 subgroup				
Genotype	Long	Short	Not typed	Non I, non II	I	II	I/II
P[4]G1	4	0	0	0	3	1	0
P[6]G1	27	9	1	0	7	22	6
P[8]G1	15	0	1	0	0	13	1
P[4]/P[8]G1	2	0	0	0	0	2	0
P[6]/P[8]G1	24	0	0	0	0	24	0
P[6]G2	3	0	1	1	1	0	0
P[4]G8	3	1	0	0	3	0	1
P[6]G8	3	1	0	0	4	0	0
P[4]/P[6]G8	6	0	0	0	6	0	0
P[4]/P[8]G8	1	0	1	0	0	0	0
P[6]G9	0	5	0	0	3	0	2
P[8]G?	1	0	0	0	0	1	0
Total (%)	89 (84.8)	16 (15.2)	4 (3.8)	1 (1.0)	27 (25.7)	63 (60.0)	10 (9.5)

in the community the stool specimens represented or whether only infants and children with severe diarrhea were taken to the clinic.

One hundred eleven (26.4%) of the stool specimens tested positive for group A rotavirus. The majority of positive specimens (92 [83%] of 111) originated from children in the 0–24 months age group, with 33% from the 0–12 months age group and 26% from the 13–24 months age group (Figure 1).

The predominant clinical symptoms reported for the children of all age groups who tested positive for rotavirus included diarrhea (100% of children), vomiting (68%), fever (48%), and nausea (48%). Nausea and vomiting were more prevalent in rotavirus-positive patients (48% of children had nausea, 68% had vomiting) than in rotavirus-negative patients (18% of children had nausea, 30% had vomiting). In contrast to the other symptoms, respiratory symptoms were recorded at similar rates in rotavirus-positive and rotavirus-negative patients (8% vs 10%) (Table 1).

Electropherotypes. The typical 4–2-3–2 grouping of the rotavirus RNA segments were seen in 105 (94.6%) of the rotavirus-positive isolates. Of these, 89 (84.8%) were the long RNA electropherotype, and 16 (15.2%) were the short RNA electropherotype (Table 2). Three distinctive patterns were seen among the long RNA electropherotypes. The short RNA electropherotypes had identical migration patterns of the RNA segments.

VP6 subgroup specificity. VP6 subgroup I strains were detected in 27 (24.3%) of the specimens, of which 16 strains had long RNA electropherotypes and 11 had short RNA electropherotypes. VP6 subgroup II strains were detected in 63 (56.8%) of the rotavirus-positive stool specimens and all had long RNA electropherotypes. Ten (9.0%) of the stool specimens showed both subgroup I and subgroup II specificity against the

Table 3. Rotavirus P and G genotypes detected in young children with diarrhea in the Vhembe Region, Limpopo Province, South Africa

	No. (%) of strains, by VP7 genotype					
VP4 genotype	G1	G2	G8	G9	Not typed	Overall
P[4]	4	0	4	0	0	8 (7.2)
P[6]	36	3	4	5	4	52 (46.8)
P[8]	15	0	0	0	1	16 (14.9)
Mixed						
P[4]/P[6]	0	0	6	0	0	6 (5.4)
P[4]/P[8]	2	0	1	0	0	3 (2.7)
P[6]/P[8]	24	0	0	0	0	24 (21.6)
Not typed	1	0	0	0	1	2 (1.8)
Total (%)	82 (73.9)	3 (2.7)	15 (13.5)	5 (4.5)	6 (5.4)	111 (100)

MAbs, of which 5 isolates were long RNA electropherotypes and 5 were short RNA electropherotypes. One specimen (0.9%) showed no reactivity with either subgroup I or II specific MAbs and had a long RNA electropherotype. Four specimens (3.6%) could not be typed with the VP6 MAbs (Table 2).

Distribution of rotavirus genotypes in Limpopo Province. The HRV strains circulating during 1998–2000 in the rural communities in the impoverished Vhembe region, RSA are presented in Table 3. The P type (VP4 associated) was successfully identified for 109 (98.2%) of the stool specimens. The P[6] genotype was the most predominant P type and was identified in 46.8% of the strains, followed by P[8] (in 14.9%) and P[4] (in 7.2%). The P[6] strains were more prevalent during 1998 (21% of specimens) and 1999 (21%) than during 2000 (6%). The P[8] strains occurred during all 3 years, ranging from 5% of specimens in 1998 to 9% in 1999 and 2% in 2000. The P[4] strains were not detected during 1998 but were seen in 1% of the specimens during 1999 and 7% of the specimens during 2000.

The G type (VP7 associated) was successfully determined in 105 (94.6%) of the stool specimens. Sequence analysis of the 2 G9 strains with short electropherotypes and VP6 subgroup I specificity indicated that these 2 strains were closely related to an MW69 human G9 strain isolated from Malawi and were therefore classified as G9 strains. G1 was the most predominant G type (73.9% of strains), followed by G8 (13.5%), G9 (4.5%), and G2 (2.7%). Only 1 rotavirus strain (0.9%) could not be assigned a P or G type.

The P[6]G1 strain was most prevalent (detected in 32.4% of specimens), followed by P[8]G1 (13.5%), P[6]G9 (4.5%), P[4]G8 (3.6%), P[4]G1 (3.6%), P[6]G8 (3.6%), and P[6]G2 (2.7%). Dual infections, with >1 P type, were seen in 37.1 % of HRV-positive specimens (Table 2). P[6]/P[8] strains were detected in 13 specimens in 1998 and 11 specimens in 1999. Dual P[4]/P[8] infections were detected in 2 specimens in 1999 and 1 in 2000. Dual P[4]/P[6] infections were detected only in 2000, in 6 specimens.

DISCUSSION

This study describes the genetic diversity of HRV strains, with identification of unusual G and P types, circulating in a rural community in the Vhembe region of the Limpopo Province, RSA, between 1998 and 2000. The study provides valuable new data regarding the HRVs circulating in rural areas of the RSA and Africa. The majority of previous studies in the RSA used diarrheal stool specimens obtained from hospitalized patients in urban and periurban areas [4, 5, 20, 21, 23, 26, 41–43]; only 1 study addressed the HRV types in a rural community from a different province, namely Kwazulu-Natal [26]. Investigations into the HRV types circulating in other African countries, namely Tanzania [44], Kenya [45, 46], Egypt [47], Malawi [48], Nigeria [49], Tunisia [50], and Guinea-Bissau [51], also focused predominantly on diarrheal specimens from urban and periurban communities, with limited studies in rural communities in Kenya [45, 46], Zimbabwe [52], Ghana [53, 54], and Gabon [55].

As has been documented elsewhere for urban, periurban, and rural children in RSA [23, 26, 42, 43], in this investigation HRV infection occurred more frequently in children <2 years of age, predominantly in infants <1 year old. Similar trends have been reported from many developing countries, namely India [56]; North African countries, such as Egypt [47] and Tunisia [50]; Central African countries, such as Malawi [48]; East African countries, such as Tanzania [44] and Kenya [45, 46]; West African countries, such as Guinea-Bissau [51] and Nigeria [49]; and other Southern African countries, such as Zimbabwe [52].

Global studies on the distribution of HRV genotypes from different geographic regions have indicated that the most prevalent strains causing childhood diarrhea worldwide are the P[8]G1, P[8]G3, P[8]G4, and P[4]G2 strains [9]. Although in this study the highly prevalent type P[8]G1 was identified in 13.5% of strains during the 3 year of surveillance, unusual types such as P[6]G1 predominated (32.4% of strains), with P[4]G1 (3.6%), P[6]G8 (3.6%), P[4]G8 (3.6%), and P[6]G2 (2.7%) occurring to a lesser extent. These findings are similar to observations reported in other African and developing countries, where the circulating HRV strains have been found to be more diverse [53, 54, 56, 57]. The appearance of these unusual types may be due to possible reassortments during natural infections [56]. In 37.1% of infections, dual genotypes were found: P[4]/P[8]G1 in 1.8% of infections, P[6]/P[8]G1 in 21.6%, P[4]/P[6]G8 in 5.4%, and P[4]/P[8]G1 in 1.8%; these findings are similar to data reported for Guinea-Bissau [57].

In this investigation the P[6] strain was the predominant VP4 type (46.8%) and occurred more frequently during 1998 and 1999. The VP4 P[6] strain is becoming more prevalent globally and was previously seen only in neonates and occasionally in older children with diarrhea in RSA [5, 25, 26]. The P[4] strains were identified in 7.2% of all rotavirus strains. No P[4] types were seen during 1998, but they reemerged during 2000, constituting 6.5% of the strains. Rotavirus P[8] strains were identified in 14.9% of the specimens and circulated during all 3 years. Mixed VP4 types, namely P[4]/P[6], were identified in 5.4% of the specimens, with P[4]/P[8] in 2.7% and P[6]/P[8] in 21.6%.

Rotavirus G1 strains are the most common circulating strain globally [9]. In this study, G1 was also the predominant G type (73.9% of strains), and the combination of P[6]G1 was the most prevalent strain (32.4%) during 1998 and 1999, followed by the P[8]G1 strain, which was identified in all 3 years of the study. Rotavirus G2 strains were identified in 2.7% of the iso-

lates and only in association with the P[6] serotype. Although the P[4]G2 strains is predominant globally, it was not detected during this study.

The G8 strain, commonly believed to be a reassortment between human and bovine rotaviruses [58, 59], has been associated with human infection in Egypt [60], Kenya [46], Guinea-Bissau [61], and in 5 patients from periurban areas in the Gauteng Province, South Africa [62]. Combinations of P[4]G8 (3.6%) and P[6]G8 (3.6%) were frequently isolated in the present study, suggesting that G8 may be more prevalent in the rural areas of the RSA. One of the P[6]G8 strains was identified as subgroup I with a short RNA electropherotype pattern, which identifies it as a strain of human origin [12]. However, the majority of G8 strains were identified as subgroup I with long RNA electropherotype patterns, which are usually associated with animal strains [59, 63–65] suggesting reassortment between animal and human strains due to the close proximity of the people with their livestock in the rural areas of RSA.

In this study, the majority (4.5%) of G9 strains were P[6]G9 with subgroup I or I/II and short RNA electropherotype patterns. This strain has also been identified in Cape Town and Pretoria [5], which suggests that it is widely in circulation in the RSA, which is similar to findings in Nigeria [66]. The emergence of G9 strains as an important cause of infantile diarrhea has been reported from many countries worldwide [53, 54, 67], and the G9 strain is currently considered to be the fifth most common globally.

Although G3 and G4 have been considered common types globally [9], no G3 or G4 strains were detected in any specimens during this study. This is similar to findings reported from Ghana [54], Nigeria [53], and India [38], which further highlights the need for continued surveillance to establish which HRV strains are circulating in a community at a given time. In addition, the sensitivity of available primers for the characterization of rotavirus strains in different geographic regions of the world needs constant monitoring. During the study, it was found that the "Gouvea" primers showed cross-reactivity between types G3 and G8, and additional RT-PCR using the "Das" primers was necessary to confirm the VP7 genotyping results [68]. The majority of G3 serotypes initially typed as a G3 with the Gouvea primers were confirmed to be G8 serotypes with the Das primers. It has been suggested that G8 strains could have originated from G3 rotavirus strains [68] which could explain the cross-reactivity noted between type G3 and G8.

Only 1 distinct short RNA electropherotype pattern was seen in this study, whereas 3 distinct long RNA electropherotype patterns were found to be circulating. Of strains with short RNA electropherotypes, 8.% were P[6]G1, 4.5% were P[6]G9, 0.9% were P[4]G8, and 0.9% were P[6]G8. Of the strains with long RNA electropherotypes, 24.3% were P[6]G1 and 13.5% were P[8]G1 (13.5%). Long RNA electropherotype subgroup II specificity is indicative of human strains [69], whereas long RNA subgroup I specificity could indicate animal strains [63, 64, 70]. However, Taniguchi and coworkers [70] proposed that long RNA subgroup I strains could be a potential new human serotype. In this study, 26 (25.0%) of the long strains showed RNA subgroup I specificity, which substantiates evidence of continued genetic interaction and evolution of rotavirus strains in rural communities living in close association with domestic animals and cattle. The occurrence of P[6]G1 and P[4]G1 (long and short electropherotypes and different subgroups) and P[6]G2, P[4]G8, and P[6]G8 strains could be ascribed to close human-animal association. The majority of P[6]G9 strains were subgroup I and had short electropherotypes. In addition 7 of the 9 P[6]G1 strains, all of which had short electropherotypes, belonged to subgroup I. This suggests possible reassortments between the P[8]G1 and P[6]G9 strains involving the VP6 gene, resulting in the high number of mixed P types (Tables 2 and 3).

The inclusion of comprehensive cross-reactive strains in a successful rotavirus vaccine will prevent infection of rotavirus in the first years of life and reduce the mortality associated with diarrhea in children [32]. This demonstration of unusual P and G combinations in a rural community of RSA may affect the efficacy of current vaccine formulations [71] and may contribute to the design and development of a broadly reactive rotavirus vaccine for use in developing countries. Therefore, surveillance of animal rotaviruses and their P and G combinations is an important aspect for future surveillance [72, 73].

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