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SUPPLEMENT ARTICLE

Rotavirus Genetic Diversity, Disease Association, and Temporal Change in Hospitalized Rural Kenyan Children

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Background. The effectiveness of rotavirus vaccines will be dependent on the immunity conferred against prevalent and emergent variants causing severe diarrheal disease. Longitudinal surveillance of disease-causing strains is a prerequisite to intervention.

Methods. Molecular characterization was conducted on rotavirus-positive stool samples from children admitted with diarrhea to a rural district hospital during 2002–2004. Extracted viral RNA was separated by polyacrylamide gel electrophoresis, and rotavirus VP4 (P types) and VP7 (G types) specificities were determined.

Results. Among 558 investigated cases, the predominant genotype was P[8]G1 (42%), followed by P[8]G9 (15%), P[4]G8 (7%), P[6]G8 (6%), and P[8]G8 (4%), with 10% mixed strains. Overall, there were 6 different P types and 7 G types. No association was identified between genotype and child age, sex, or severity of diarrhea. The P and G genotypes and polyacrylamide gel electropherotypes showed significant temporal variation in frequency: P[8]G1 decreased from 51% (95% confidence interval [CI], 43%–58%) in 2002 to 30% (95% CI, 24%–37%) in 2004, and P[4]G8 increased from 2% (95% CI, 0%–5%) in 2002 to 13% (95% CI, 9%–19%). Quarterly data revealed seasonally endemic and emergence and/or decay patterns.

Conclusions. Our study of rotavirus strains causing severe diarrhea in rural Kenyan children showed a predominance of P[8]G1 and confirms the importance of G8 and G9 strains in sub-Saharan Africa. Considerable genetic diversity of rotavirus strains was observed, including substantial mixed and unusual types, coupled with significant temporal strain variation and emergence. These results warn of variable vaccine efficacy and the need for long-term surveillance of circulating rotavirus genotypes.

Rotavirus is the leading cause of acute severe diarrhea in children <5 years of age and is estimated to be a major cause of childhood death in the developing world, in particular, the Indian subcontinent and sub-Saharan Africa [1, 2]. Group A rotavirus strains, which account for the vast majority of human disease, are classified into different P and G types on the basis of the 2 immunodominant outer capsid proteins, VP4 and VP7, respectively. A total of 28 P types and 20 G types [3–6] have been identified worldwide, with P[8]G1, P[8]G3, P[8]G4, P[4]G2, P[8]G9, and P[6]G9 frequently detected in annual epidemics [7].

Studies have shown wide geographical variation in the prevalence of G and P types across continents and local and global temporal changes in the frequency of dominant strains and emergence of unusual P and G types and combinations [7]. Globally, 85% of rotavirus strains studied carry P[8]G1, P[4]G2, P[8]G3, or

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P[8]G4 specificity. However, in South America, the proportion of the common genotypes is 68%, and in Africa, only 50% of strains belong to one of these genotypes. Furthermore, studies in Africa show only 23% of strains with P[8]G1 (compared with 65% worldwide) and significant proportions of P[6], G8, unusual, and mixed infections [7–15].

During the past 15 years, G9 strains have emerged as an important disease-causing variant, and recently, genotype G12 strains have also emerged globally in a manner similar to the emergence of G9 strains in the early 1990s [7]. In Africa, both of these genotypes have been detected, and G9 strains have been shown to predominate in certain settings, replacing the more traditional genotypes for 1–2 seasons or circulating at low background levels over a number of seasons. In addition, the G9 genotype has been found in combination with P[6] or P[8] VP4 specificity, subgroup I or subgroup II VP6 specificity, and either long or short electropherotypes, suggesting a promiscuous nature and a predilection for reassortment [16, 17].

Assessment of the impact of a rotavirus vaccine should take into account the natural temporal variability in P and G types. The compositions of the 2 live oral rotavirus vaccines, Rotarix (GlaxoSmithKline) and RotaTeq (Merck), present very different immunization strategies, with the former based primarily on heterotypic immunity and the latter based more on homotypic immunity [18, 19]. Although both have demonstrated safety and proved to be efficacious in various developed and developing countries, vaccine effectiveness after implementation will have to be closely monitored. In this way, any influence on vaccine efficacy by the emergence and temporal increase in the frequency of new variants, both naturally occurring and induced through the process of genotype replacement, can be determined. Longitudinal genetic surveillance of disease-causing rotavirus strains is a clear priority, particularly in Africa before and after wide-scale vaccine introduction.

MATERIALS AND METHODS

Study samples. The present study used rotavirus-positive stool samples from surveillance of pediatric (age, 0–12 years) patients admitted to Kilifi District Hospital in coastal Kenya from the period January 2002 through December 2004 [20]. Of 2039 patients with diarrhea and 620 control subjects without diarrhea, 588 (29%) and 19 (3%), respectively, were group A rotavirus antigen positive, as determined by enzyme immunoassay (DAKO Rotavirus IDEIA; Oxoid). Contemporaneous control subjects were selected from children admitted without a history of diarrhea frequency matched by age to patients at a ratio of 1:3. Samples were stored unprocessed at −80°C before being shipped on dry ice to the Medical Research Council–Diarrhoeal Pathogens Research Unit, South Africa, for further analysis. The present study performed molecular characterization of 558 (95%) and 12 (63%) group A rotavirus isolates obtained from patients with or without severe diarrhea, respectively.

Life-threatening diarrhea was assigned to a case with ⩾1 of the following clinical features: hypoxia (<90% saturation), prostration or coma, hyponatremia, hypoglycemia, severe malnourishment, or bacteremia. Full details of the sampling and testing procedures, clinical definitions, and the sociodemographic, clinical, and laboratory characteristics of the children are reported elsewhere [20]. The Kenyan National Research Ethical Committee and the Coventry Research Ethics Committee, United Kingdom, granted ethical approval for the study.

Polyacrylamide gel electrophoresis (PAGE). The double-stranded RNA (dsRNA) genome was extracted from 10% fecal suspensions with use of the standard phenol-chloroform method, followed by ethanol precipitation in the presence of 0.3 mol/L sodium acetate. The extracted RNA was resolved on 10% polyacrylamide gels with 3% stacking gels with use of a discontinuous buffer system at 100 V for 18 h at ambient temperature [21]. RNA segments were then stained by silver nitrate according to published methods [22].

Viral RNA extraction for reverse-transcription polymerase chain reaction (RT-PCR). Viral dsRNA was extracted from 250 μL of rotavirus-positive fecal suspensions with use of Tri-Reagents-LS (Molecular Research Centre) according to the manufacturers' instructions. The extracted dsRNA was further precipitated in ice-cold isopropyl alcohol, and the air-dried pellet was resuspended in 40 μL of deionized water.

RT-PCR. The extracted dsRNA was subjected to RT-PCR using several primer pairs taken from highly conserved regions of the RNA genome. The primer pair sBeg/End9 was used to generate full-length copies of the VP7 gene (1062 base pairs [bp]), and 9con1/EndA was used to generate 903 bp of VP7 gene fragments in specimens that were difficult to type [23–25]. Con2/Con3 and VP4F/VP4R primer pairs were used to amplify VP4 gene fragments of the 876 bp and 663 bp, respectively [26, 27]

Rotavirus typing. Genotyping of the cDNA was performed by nested multiplex PCR using type-specific VP7 and VP4 primers described elsewhere [10, 23, 25, 26, 28–30]. The nontypeable G and P genotypes were further analyzed using the animal G and P primers described by Gouvea et al [31, 32]. Combinations of P and G genotypes have been defined as usual or unusual on the basis of delineations described by Santos and Hoshino [7].

Statistical analysis. Data were analyzed using Stata, version 10.1 (Statacorp). Exact 95% confidence intervals were defined for genotype frequencies. The χ^2 test was used to test for lack of independence in cross-tabulations (ie, for possible association between 2 variables), pooling marginal totals of <15%, and the Wilcoxon rank-sum test was used for equality of distributions.

RESULTS

Among 558 group A rotavirus–positive diarrhea cases (median patient age, 10 months [interquartile range, 7–15 months]; 60% male), 82 were PAGE negative, 26 were G nontypeable, 30 were P nontypeable (including 6 both G nontypeable and P nontypeable), and 508 were positive for G and P types. Of 12 group A rotavirus-positive nondiarrhea cases in control subjects (median age, 25 months [interquartile range, 2–39 months]; 42% male), 2 were negative for PAGE and P type.

PAGE distribution in diarrhea cases. Of 476 rotavirus strains, 86 (18%) and 390 (82%) displayed short and long electropherotypes, respectively (Table 1, which appears only in the electronic version of the *Journal*). Three long electropherotypes (L1, L2, and L3) were detected in 63%, 11%, and 4.4% of cases, respectively, and short electropherotypes (S3, S2, and S1) were identified in 8%, 4.6%, and 3.6% of cases. The remainder of the electropherotypes detected (L4–6, mixed, and S4) had prevalences of <2%. There was evidence of temporal variation in the PAGE composition among the 3 years, in particular, L1 and L2 and S3 types ($\chi^2_{(10)}$, 123.681; $P < .001$) (Table 1, which appears only in the electronic version of the *Journal*). The proportion of PAGE-negative strains did not change by year ($\chi^2_{(2)}$, 3.493; $P = .174$).

Genotype distribution in diarrhea cases. Among the 558 cases analyzed for both VP4 (P) and VP7 (G) genes, 6 different P types and 7 G types were identified in both single and mixed infections (Tables 2 and 3). Among the P types, P[8] was predominant (68% of all samples), followed by P[6] and P[4] (both 12%); the other types (P[9], P[11], and P[14]) had a low prevalence and were present in only mixed infections (Table 3). Among the G types, G1 predominated (48%), followed by G9 (19%) and G8 (18%); the remainder (G2, G3, G4, and G10) had a low prevalence, and G3, G4, and G10 were present as only mixed infections (Table 3).

The predominant P-G genotype was P[8]G1 (detected in 42% of cases), followed by P[8]G9 (15%), P[4]G8 (7%), P[6]G8 (7%), and P[8]G8 (4%). Usual and unusual genotype combinations were identified in 61% and 20% of isolates, respectively, or if excluding mixed isolates, in 68% and 25%, respectively (Table 2). The remaining single genotypes occurred at low frequency. Mixed infections occurred in 57 cases (10%), with 56% of these involving P[8] and 32% including P[8] in combination with G1 (Table 2). Specimens untypeable for at least 1 of VP4 or VP7 genes occurred with a frequency of 9% (Table 2).

Table 1. Rotavirus Polyacrylamide Gel Electrophoresis (PAGE) Types in 558 Pediatric Diarrhea Cases at Kilifi District Hospital, Kenya, 2002–2004

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases*

Table 2. P and G Combinations for 558 Group A Rotavirus-Positive Pediatric Diarrhea Cases at Kilifii District Hospital, Kenya, 2002–2004

	No. (%) of cases			
Type	2002 (n = 178)	2003 (n = 169)	2004 (n = 211)	Total (n = 558)
Usual				
P[4]G2[x]	1 (1)	4 (2)	0 (0)	5 (1)
P[6]G1	3 (2)	0 (0)	6 (3)	9 (2)
P[6]G9[xq]	3 (2)	4 (2)	5 (2)	12 (2)
P[8]G1	90 (51)	79 (47)	63 (30)	232 (42)
P[8]G9	23 (13)	36 (21)	25 (12)	84 (15)
Subtotal	120 (67)	123 (73)	99 (47)	342 (61)
Unusual				
P[4]G1	0 (0)	0 (0)	10 (5)	10 (2)
P[4]G8[xq]	3 (2)	7 (4)	28 (13)	38 (7)
P[4]G9[xq]	0 (0)	2 (1)	1 (0)	3 (1)
P[6]G8[xq]	17 (10)	1 (1)	15 (7)	33 (6)
P[8]G2	4 (2)	1 (1)	1 (0)	6 (1)
P[8]G8	8 (4)	1 (1)	13 (6)	22 (4)
Subtotal	32 (18)	12 (7)	68 (32)	112 (20)
NT or mixed				
P[4]GNT[xq]	0 (0)	0 (0)	6 (3)	6 (1)
P[6]GNT[xq]	2 (1)	0 (0)	3 (1)	5 (1)
P[8]GNT	8 (4)	0 (0)	1 (0)	9 (2)
P[NT]G1	5 (3)	5 (3)	2 (1)	12 (2)
P[NT]G8[xq]	1 (1)	1 (1)	1 (0)	3 (1)
P[NT]G9[xq]	2 (1)	2 (1)	2 (1)	6 (1)
P[NT]GNT	1 (1)	2 (1)	3 (1)	6 (1)
Mixed	7 (4)	24 (14)	26 (12)	57 (10)
Subtotal	26 (15)	34 (20)	44 (21)	104 (19)

NOTE. NT, nontypeable.

Genotype association with age, sex, or disease severity. Data on genotypes with cases numbering <10%–15% were pooled for analysis. In a comparison of 350 infants (age, <1 year) with 208 children (age, 1–5 years), there was no significant difference in the distribution of genotypes ($\chi^2_{(6)}$, 3.745; $P = .711$). Similarly, the distribution of genotypes in 334 male individuals, compared with 224 female individuals, did not significantly differ ($\chi^2_{(6)}$, 5.992; $P = .424$). There was no association between genotype and presence ($n = 119$) or absence ($n = 439$) of life-threatening diarrhea ($\chi^2_{(6)}$, 4.453; $P = .616$).

Temporal variation in genotype distribution. The distribution of rotavirus genotypes by year is shown in Tables 2 and 3. After pooling low-frequency observations (<15 cases over the 3 years), there was evidence for significant temporal variation in P-G genotype composition ($\chi^2_{(12)}$, 72.841; $P < .001$); in particular, there was an increase in P[4]G8 from 2% in 2002

Table 3. P-G Type Combinations in 57 Mixed Infections in Pediatric Patients with Diarrhea at Kilifii District Hospital, Kenya, 2002–2004

P-G type	No. of infections		
	2002	2003	2004
P[4]G1,8	0	1	2
P[4]G1,9	0	1	1
P[4]G1,10	0	1	0
P[4]G8,9	0	0	1
P[4,6]G8	0	0	2
P[4,8]G1	1	0	0
P[4,11]G4	0	0	1
P[4,11]G8	0	1	0
P[4,14]G2	1	0	0
P[6]G1,2	0	0	1
P[6]G1,8	0	1	1
P[6]G1,9	0	1	1
P[6]G8,9	0	0	1
P[6,8]G1	0	1	0
P[6,8]G9	0	0	2
P[6,8]G1,8	0	1	0
P[6,8]G2,8,3	0	1	0
P[6,8,9]G9	0	0	1
P[9]G1	0	0	1
P[8]G1,2	1	1	0
P[8]G1,8	0	9	9
P[8]G1,9	2	2	1
P[8]G8,9	0	2	0
P[8,9]G1	1	0	0
P[NT]G1,8	0	1	1
P[NT]G1,9	1	0	0
Total	7	24	26

NOTE. NT, nontypeable.

to 13% in 2004 and a decrease in P[8]G1 from 51% in 2002 to 30% in 2004 (Figure 1). Other genotypes (eg, P[6]G8) showed significant fluctuation. Genotype observations stratified by year and quarter (Figure 2) provide further detail of the temporal variation, including seasonal patterns (P[8]G1), emergence (P[4]G8), and decay and reemergence (P[6]G8). The diversity of single genotype infections was roughly constant at 9 different genotypes during 2002–2003 and 10 genotypes in 2004 (Table 2). However, as shown in Figures 2 and 3 (which appears only in the electronic version of the *Journal*), there was a decrease in the dominance of one (primarily P[8]G1) or a few genotypes coupled with altered seasonality in total diarrhea admissions and rotavirus cases during 2004, compared with 2002–2003.

During 2004, an increase in G8 strains in combination with P[4], P[6], and P[8] was noted (Table 2). Investigation of mixed infections during 2003 provided possible evidence for human-human reassortment events, because P[6]P[8]G1G8, P[6]P[8]G2G8G3, and P[4]P[6]G8 infections were detected. In addition, the increase in P[4]G1 infections in 2004 may have resulted from mixed infections with G8, G9, and G10 in 2003. Additional analysis of the full genomes of unusual human-human reassortants and investigation of the population of rotavirus strains causing mixed infections will be required.

Control subjects. Among the 12 cases in positive control subjects with genetic results, 7 were L1; 3 were L2; 4 were P[8]G1, 2 P[8]G9, and 2 P[nontypeable]G1; and 4 were mixed (3 P[8]G1,9 and 1 P[8]G8,9)—approximately corresponding to the distribution in cases. The asymptomatic control subjects were noticeably older than the symptomatic patients (age, 25 months vs 10 months).

Vaccine type prevalence. Of 558 cases, 432 (77%) and 439 (79%) had types in common with those in the Rotarix (P[8]G1) and RotaTeq (P[5]G1–4 and P[8]G6) vaccines, respectively. The proportions not represented in Rotarix were 29 (16%) of 178, 20 (12%) of 169, and 70 (33%) of 211 during 2002, 2003, and 2004, respectively ($\chi^2_{(2)}$, 24.291; $P < .001$). Correspondingly, the proportions not represented in RotaTeq were 31 (17%) of 178, 24 (14%) of 169, and 71 (34%) of 211 ($\chi^2_{(2)}$, 29.420; $P < .001$).

DISCUSSION

We investigated the genetic characteristics of group A rotavirus strains in clinically well-defined severe pediatric diarrhea cases in a rural Kenyan setting over a 3-year period. The study revealed no evidence of an association between genotype and age, sex, or disease severity. However, we identified considerable genotypic diversity, with patterns of prevalence both consistent

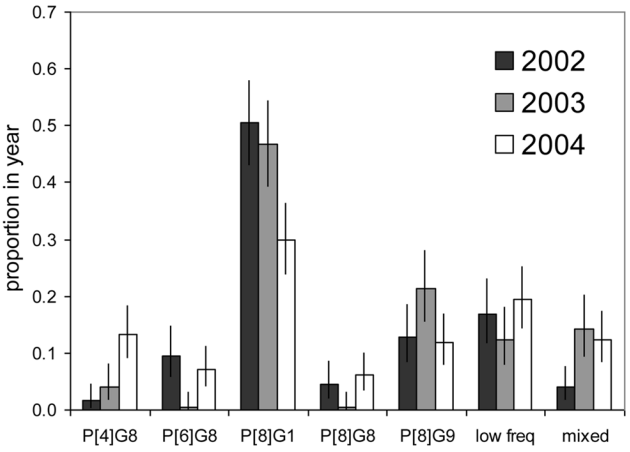

Figure 1. Variation by year (2002–2004) in P-G genotype frequencies (with exact 95% confidence intervals) determined for 558 rotavirus-positive samples from Kilifi District Hospital, Kenya. Low-frequency types include all single-strain genotypes with a frequency over the 3 years of <15 cases.

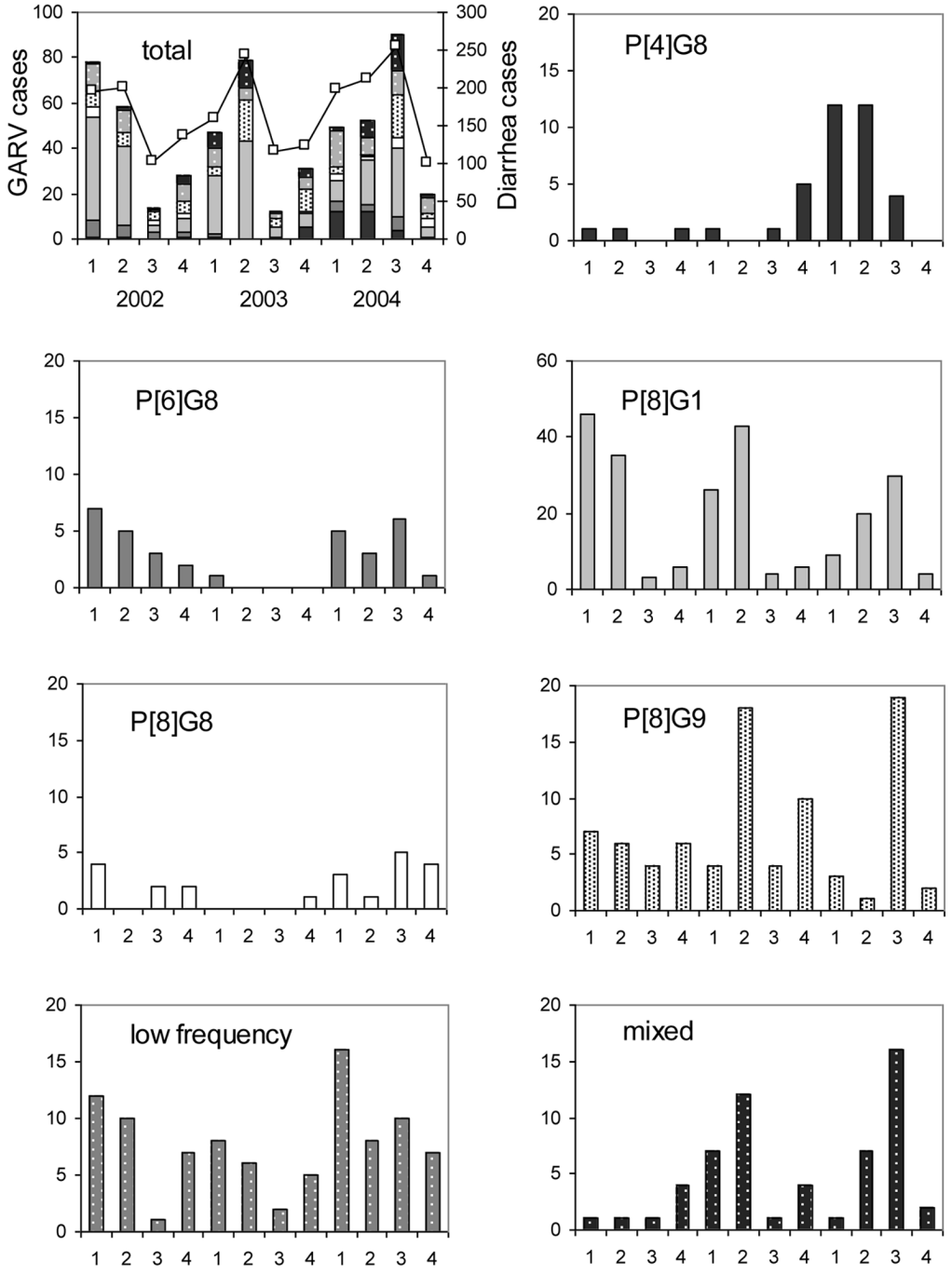

Figure 2. Seasonal variation in P-G genotypes determined for 558 group A rotavirus (GARV)–positive samples from Kilifi District Hospital, Kenya (2002–2004). The top left panel details totals for all variants by year and quarter *(bars)* and total cases of diarrhea *(line)* from which GARV-positive cases were identified.

and in contrast to those elsewhere in Africa. Similar to Africa in general and Kenya specifically, we found a predominance of genotype combinations G1, G8, and G9, with P[4], P[6], and P[8] [7, 11]. The G-P combinations found in >90% of isolates in temperate countries in Europe and North America and in parts of Australia and Oceania were identified at a proportion of only 68%, consistent with the African context [7]. However, the prevalence of P[8]G1 (42%) and P[8]G9 (15%) was unusually high, there was an absence of 2 usually frequent genotypes (P[8]G3 and P[8]G4), and no case of the emergent

This figure is available in its entirety in the online version of the *Journal of Infectious Diseases.*

Figure 3. Distribution of genotype frequencies by month during 2002 *(A),* 2003 *(B),* and 2004 *(C)* among 558 rotavirus-positive patients admitted to Kilifi District Hospital, Kenya.

G12 type was identified. Genotypes recognized as unusual worldwide [7], in particular, G8 variants (P[4]G8, P[8]G8, and P[6]G8), comprised 25% of all single strains (Table 2), and the prevalence of mixed G-P combinations was 10%; both proportions were similar to that in Africa overall [7].

Furthermore, we observed significant temporal variation in the prevalence of some genotypes. Although P[8]G1 was the predominant variant overall, its prevalence decreased markedly from ~50% during 2002–2003 to 30% during 2004. By contrast, the prevalence of P[4]G8 increased from 2%–4% during 2002–2003 to 13% during 2004, and the prevalences of both P[6]G8 and P[8]G8 were moderate during 2002 and 2004 and were almost absent during 2003. Of further interest, mixed genotypes showed a significant increase between 2002 and 2003–2004. Temporal variation of this nature has been observed previously [33]. Kenya, for example, has seen the emergence of G8 and G9 and a shift in predominance of P[8] to P[6], accompanied by considerable year-to-year variation [34]. However, our data show local patterns inconsistent with the national picture; for example, P[8] remains the predominant P type in Kilifi for all years studied. All these studies show the limitation of short-term surveillance in discerning patterns of prevalence; however, in addition, our results show the importance of widespread monitoring to capture local variation, which may be of importance in assessing vaccine impact.

Rotavirus hospitalizations are often observed to have marked seasonality, particularly in developed countries. In Kilifi, we observed peak group A rotavirus admissions from March through May during 2002–2003, with a higher prevalence during 2004, particularly during the third quarter, all of which mirrored total diarrhea admissions over this period (Figure 2) [20]. A greater proportional representation of a wide group of variants occurred during 2004 (Figure 2), with the emergence of P[4]G8 early in 2004 associated with a delay in the peak in P[8]G1 to the third quarter. Clearly, longer-term surveillance has the capacity to identify genotype emergence, decay, and altering dominance patterns, which have a bearing on potential vaccine effectiveness and the value of before–and-after studies in assessing vaccine impact.

The diversity of group A rotavirus types identified in Kilifi is supplemented by a high proportion of unusual G-P combinations, among which are likely reassortant strains. These include rare occurrences of P[4]G9 and P[8]G2 and emergence of P[4]G1, all probable human-human reassortants. The presence, among isolates of mixed G-P type, of strains inclusive of these unusual and emergent combinations (eg P[4]G1,8; P[4]G8,9; P[6,8]G1,8; P[6,8]G2,3,8; P[8]G1,8; and P[8]G8,9) provides strong circumstantial evidence for natural reassortant events arising from cocirculating local strains. The prevalence of 9% of strains untypeable for at least one of VP4 or VP7 genes, presumably a result of antigenic drift, provides further evidence for extensive natural variation in rotavirus strains in this location.

Esona et al [35] recently investigated the characteristics of all 11 genes of G8 rotavirus strains detected in Cote d' Ivoire, Cameroon, Ethiopia, and Tunisia. The study found that genes for VP7, NSP2, and NSP5 were closely related to cognate genes of animal strains and suggested that African G8 strains may have arisen through reassortment of VP7 and VP4 genes. In our study, large numbers of P[4]G8 (emerging in 2004) and P[6]G8 and P[8]G8 (showing marked year-to-year variation) were detected. Furthermore, G10, P[11], and P[14] genotypes, more commonly associated with infections in animals, were detected in mixed infections. Although additional analysis of the full genomes of these strains will be required, the possibility exists that these strains are human-animal reassortants and may provide evidence of animal rotaviruses acting as reservoirs for one or several genes of human rotavirus strains.

Severe rotavirus disease represents a major burden in developing countries and is an identified target for vaccine intervention [36]. Antigenic diversity of strains in cocirculation, antigenic drift, and emergence of new variants through reassortment (human with human and human with animal) and animal introductions [33] represent considerable potential for impaired vaccine efficacy. Our study provides support for the presence of each of these elements of variation in Kilifi, which, together with evidence of geographical and temporal variation in prevalent genotype composition, have a bearing on potential vaccine effectiveness and on the potential to measure vaccine impact through surveillance.

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