

Original Research Article

Diversity of Rotavirus VP7 and VP4 Genotypes Associated with Severe Childhood Diarrhea in North West Cameroon: Detection of Unusual strains G1P[6], G2P[6], G2P[8] and G3P[6]

ABSTRACT

Aims: To determine rotavirus genotypes and distribution among children 0 - 5 years old with severe acute diarrhea in the North West Region (NWR) of Cameroon

Study design: Cross sectional

Place and Duration of Study: Hospitals and health centers in the NWR served as sample collection centers between January and December 2004.

Methodology: Fifty-six Enzyme Immunoassay (EIA) and Polyacrylamide gel electrophoresis (PAGE) - positive rotavirus stool specimens were analyzed for VP7 and VP4 genotypes by reverse transcriptase polymerase chain reaction (RT-PCR).

Results: A total of 51 (91.1%) samples genotyped as G and P types while the remaining five samples (8.9%) were partially characterized. Four VP7 genotypes (G1, G2, G3 and G9) and two VP4 genotypes, P[6] and P[8] were detected. The predominant G and P types were; G3 (34%) and P[8] (41.1%). Genotypes G1-G3 accounted for 85.8% of isolates while G9 represented 3.6% of isolates. Genotype P[6] represented 30.4% of all VP4 genotypes and was the most widespread strain occurring in all age groups. Twelve single G and P-type combinations were identified. Genotype G1P[8] (19.6%) predominated, followed by G3P[6] (17.9%). Unusual strains detected were G1P[6], G2P[6], G2P[8] and G3P[6] accounting for 32% of cases. Mixed infections were detected from 15 (27%) isolates comprising G1/G3P[6], G2/G9P[6], G2P[6]/P[8] and G1/G3/G8 P[6]/P[8]. Twenty isolates (35.7%) had unusual genotype / electropherotypes combinations. All G9 strains, mixed G genotypes and strains with atypical electropherotypes occurred in children hospitalized with severe gastroenteritis.

Conclusion: There is high incidence of unusual rotavirus strains circulating in the NWR of Cameroon that could have an impact on rotavirus vaccine performance. Future studies will investigate post vaccine prevalence and characterization of non-typeable strains by other methods.

Keywords: Diarrhea, Gastroenteritis, Rotavirus, genotypes, Cameroon, Central Africa

1. INTRODUCTION

Rotavirus-associated diarrhea accounts for about 500,000 deaths yearly in children under years of age worldwide with about 85% of cases occurring in developing countries of Asia and Sub-Saharan Africa [1].

Rotavirus, a genus of the family Reoviridae, has 11 segments of double-stranded RNA as its genome which are enclosed in a triple-layered capsid. These segments encode six structural proteins (VP1-VP4, VP6 and VP7) and six nonstructural proteins (NSP1-NSP6) [2]. Two outer capsid proteins, VP7 and VP4, contain neutralizing epitopes which define serotypes of rotavirus and form the basis for a dual classification of rotaviruses into VP7 (G types) and

24 VP4 (P types) genotypes [3]. Based on diversity of the VP7 and VP4 genes, G and P
25 genotypes have been defined for group A rotaviruses (RVA), respectively, and at least 27 G
26 types and 37 P types have been described [4]. In human rotaviruses, G1, G2, G3, G4, G9,
27 and G12 usually combined with P[4], P[6], and P[8] and are frequently detected throughout
28 the world, with G1P[8] being the most prevalent strain in humans [5].

29 In order to assess vaccine efficacy rotavirus strain surveillance studies are needed prior to
30 introduction of vaccines in any community [6]. There are very few studies on rotavirus in
31 Cameroon and the Central Africa region. Reports of rotavirus studies in the West and South
32 West Regions of Cameroon showed a wide diversity of strains including unusual genotypes
33 [7]. It was therefore, necessary to determine the circulating strains in the North West Region
34 (NWR) where no such studies have been conducted before the introduction of rotavirus
35 vaccines. Following the World health Organization recommendations to participating nations
36 especially those with high diarrhea related infant mortality Cameroon became the 21st
37 country to introduce rotavirus vaccines in the Expanded Program on Immunization in 2014.
38 This study was supposed to provide baseline data on the diversity and distribution of
39 rotavirus genotypes circulating in the NWR of Cameroon before the introduction of rotavirus
40 vaccines in the country.

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42 **2. MATERIAL AND METHODS**

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44 A total of 534 diarrheic stool specimens were collected from children less than 5 years of
45 age from hospitals in six health districts in the NWR and screened for rotaviruses by Enzyme
46 Immunoassay (EIA). Viral RNA was extracted from 56 EIA positive samples by Bender
47 method [8] with slight modification for polyacrylamide gel electrophoresis (PAGE) analysis.
48 The presence of rotavirus in the stool specimens was determined by detection of 11 RNA
49 segments of rotavirus by PAGE as previously described [9]. Double-stranded RNA (dsRNA)
50 was extracted from all PAGE positive specimens by sodium dodecyl sulphate and Phenol/
51 Chloroform method as described [10] and purified with the RNaid® Kit (Bio 101, Carlsbad,
52 USA).

53 **2.1. Genotyping of Rotavirus isolates**

54 Rotavirus G and P types were determined by reverse transcription polymerase chain
55 reaction (RT-PCR) as previously described [11, 12]. For VP7 (G) and VP4 (P) genotype-
56 specific amplification in the multiplex PCR (second-round PCR) primer set A (RVG9, aBT1
57 (G1), aCT2 (G2), aDT4 (G4), aET3 (G3), aAT8 (G8), aFT9 (G9) [11] and primer set B (Con3,
58 1T-1) (P[8]), 2T-1 (P[4]), 3T-1 (P[6]), 4T-1 (P[9]), 5T-1 (P[10]) [12] were used for all the
59 rotavirus-positive specimens.

60 **2.2. Statistical analysis**

61 Odds ratios with their 95% confidence intervals (CI) were calculated to establish
62 associations between age and rotavirus genotypes using EPI- Info version 3.

63

64 **3. RESULTS AND DISCUSSION**

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66 **3.1. VP7 and VP4 genotypes**

67 First and second round products including single and mixed reactivity were obtained in 45
68 (80%) and 54 (96.4%) for VP7 gene and in 52 (92.9%) and 53 (94.6%) for VP4 gene
69 respectively. A total of 54 (96.4%) and 53 (94.6%) isolates were assigned a G and P

genotype respectively. Four VP7 genotypes were identified: G1 (30%), G2 (21%), G3 (34%) and G9 (3.6%) all accounting for 91.1% of cases. With respect to G types mixed infections (G3/G1, G3/G2, G3/G1/G8 and G2/G9) were seen in five (8.9%) cases. Two P genotypes were identified: P[6], 17/56 (30.4%) and P[8] 23/56 (41%) both accounting for 71.4% of isolates. Mixed P types consisting solely of P[6]/P[8] were detected in 13 (23.2%) isolates. G3 (37.5%) and P[8] (37.5%) were the most common genotypes among 40 isolates analyzed from hospitalized children (Table 1).

Although G1 is usually the predominant G type in worldwide studies [5, 13] G3 predominated in the NWR of Cameroon. The predominance of this strain in the absence of G4 and P[4] also contrasts with reports in the neighboring West and South West regions where both genotypes were detected at low levels [7]. The prevalence of rotavirus genotypes vary from season to season and between different geographic regions [14] which could account for the differences observed. Four rotavirus genotypes (G1-4 and G9) generally constitute about 80-90% of strains detected in surveillance studies but the increasing reports of unusual strains could impact their global epidemiologic significance. A typical example is the increasing prevalence of P[6] in clinical infections [5]. High prevalence of G3, P[6] and mixed P types involving P[6]P[8] are common in this region and have been reported at high levels in Ghana, Nigeria and many parts of Africa [13]. Continuous detection of high levels of P[6] genotypes in clinical specimens may imply strain evolution with increasing pathogenicity or simply due to increasing rotavirus surveillance especially in developing countries. The strain was initially associated with asymptomatic neonatal infections but now causes significant clinical infection in all age groups representing about 33% to 50% of rotavirus disease cases [5, 15]

Infections with strains showing mixed G and P types are not uncommon in Africa. Earlier studies reported significant levels of mixed infections in Guinea Bissau [16] and other parts of the Continent [13]. Apart from increasing the risk of reassortment they are also detected more in patients with more severe disease requiring hospitalization. Although it is not clear why mosaic strains are prevalent in some regions it is possible that co-infections with other disease agents and malnutrition that weaken host defense against reinfection may be the mechanism promoting multiple infection and clinical disease manifestation. Common environmental factors such as overcrowding, mass movement and poor living conditions that are prevalent in many developing countries could also facilitate disease transmission leading to mixed infections.

Table 1. Rotavirus genotypes in children hospitalized with gastroenteritis (2004).

Genotype	P[6]	P[8]	P[6]P[8]	Pnt	Total	Percent
G1	2	5	2	0	9	22.25
G2	0	4	4	0	8	20
G3	8	5	0	2	15	37.5
G9	0	0	2	0	2	5
G1G3	0	0	0	1	1	2.5
G2G3	0	0	1	0	1	2.5

G2G9	1	0	0	0	1	2.5
G1G3G8	0	0	0	0	1	2.5
Gnt	1	1	1	0	2	5
Total	12	15	10	3	40	100

^a Three cases with short electropherotypes; *b* Short electropherotype;
Gnt = Non-typeable VP7 genotype; *Pnt* = Non-typeable VP4 genotype

3.2. Age distribution

A total of 54 (96.4%) cases of all G and P types occurred in children less than 24 months of age (Figure 1). Genotype G1 was not detected from children less than 6 months while P[6] circulated in all age groups. The odds for any age group having a specific genotype compared with other age groups were: 0 – 6 months, G2 (OR = 2.0; CI: 0.22 - 15.95) and G3 (OR = 1.13; CI: 0.30 - 15.37); 7 – 12 months, G1 (OR = 1.67; CI: 0.36 - 6.72); 13-18 months, mixed infections (OR = 14.33 CI: 1.1 - 405.5); 7 – 12 months, mixed P - types (OR = 1.84; CI: 0.44 - 8.3); 7 – 12 months, mixed P genotypes (OR = 1.84; CI: 0.44 - 8.3). P[8] (OR = 1.79; CI: 0.12 - 53.18) and non-typeable P genotypes (*Pnt*) (OR = 1.22; CI: 0.37 - 4.1) were more likely to occur in male children who are generally more active than females. Peak rotavirus infection was previously reported in ages 7 – 12 months old [16] and this age group also had age highest diversity of both G and P genotypes. Mixed genotypes were seen mainly in children less than 24 months of age defining the greater susceptibility of younger children to rotavirus diarrhea.

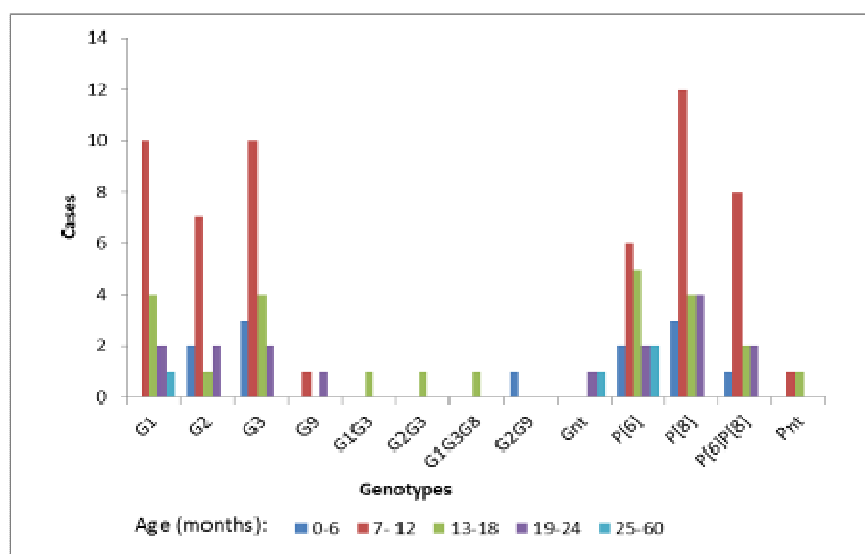


Fig. 1. Age (months) distribution of rotavirus VP7 and VP4 genotypes North West Cameroon (2004).

Gnt = Non-typeable VP7 genotype; *Pnt* = Non-typeable VP4 genotype

3.3. Monthly Distribution of VP7 and VP4 Genotypes

Figure 2 shows the monthly distribution of genotypes. Most strains circulated between December, January and February which is characteristic of most rotavirus infections [7, 13, 15]. A majority of G2 strains (75%), P[6] (55.6%) and mixed genotypes circulated mostly in January. Many studies have shown wide geographical variation in the prevalence of G and P types across continents as well as local and global temporal changes in the frequency of dominant strains [5, 13]. Knowledge of the seasonal distribution of rotavirus genotypes is important for strategic planning and effective control measures. However our data was collected only for a period of one year and the results might not reflect the true seasonality of strains but could nevertheless shed light on point prevalence of genotypes.

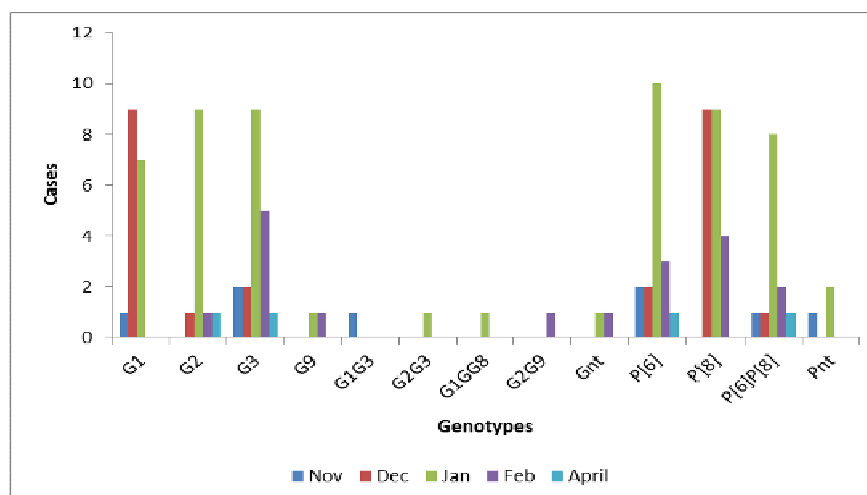


Fig.2. Monthly Distribution of Rotavirus VP7 and VP4 Genotypes Cameroon (2004).
Gnt = Non-typeable VP7 genotype; *Pnt* = Non-typeable VP4 genotype

3.4. G/P genotype combination analysis

A binary characterization was possible with 51 (91.1%) isolates comprising 12 G/P constellations with six single and six mixed G/P infections representing 36 (64.3%) and 15 (26.8%) isolates respectively (Figure 3). Genotype G1P[8] was the most common strain followed by G3 P[6] and G3P[8]. Mosaic strains with either mixed G or P genotypes (G1P[6]P[8], G2P[6]P[8], G3G2P[6], G9G2P[6]) and dual G and P reactivity, G1G3G8P[6]P[8] were seen in 15 (27%) cases. Isolates with unusual G/P combinations (G1P[6], G2P[8], G2P[6] and G3P[6]) accounted for 18/56 (32%) cases while five (8.9%) isolates were partially characterized.

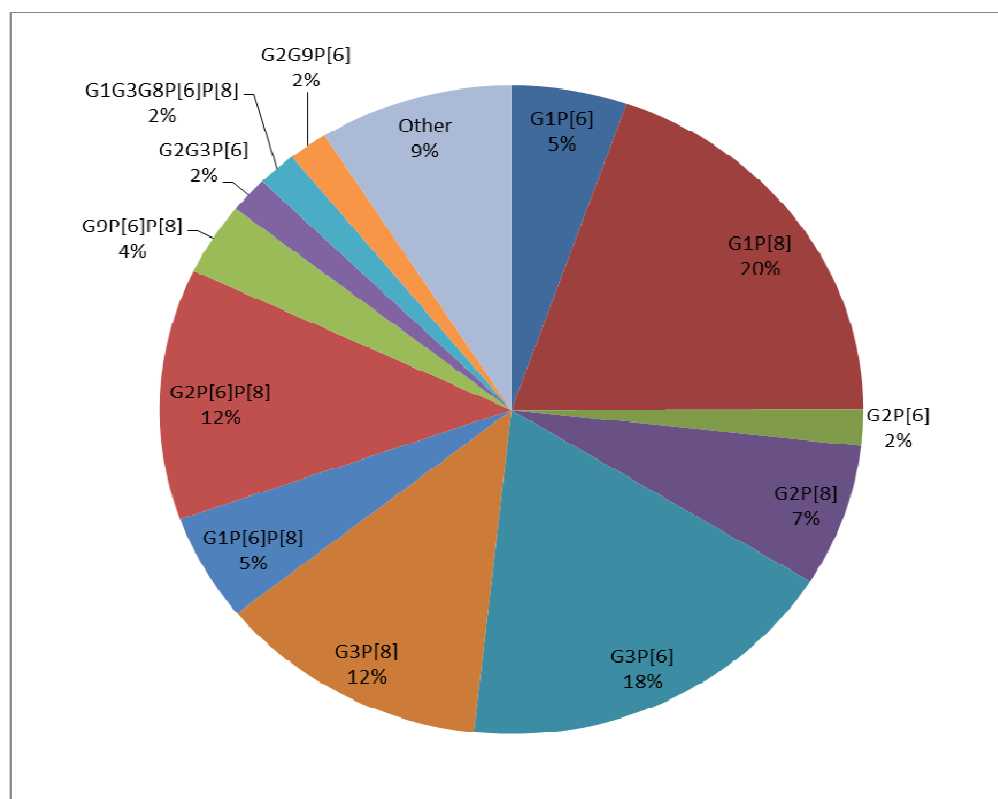
West Africa is known to show great diversity in rotavirus strains and unusual genotypes as well as mixed infections [7, 13, 18]. Therefore the detection of strains with dual reactivity to either G or P types was not unexpected. However, the detection of a strain with multiple reactivity showing both VP7 and VP4 genotype specificities (G1/G3/G8 P[6]/P[8]) was rather unique in that such combinations have not been previously reported to the best of our knowledge. The mixed reactivity in this case could have resulted from co-infection with more than one mosaic strains with long (possibly G1P[8]) and short electropherotypes (possibly G2P[6], G2P[8], G9P[8], G9P[6], or G2/G9P[6]/P[8]) based on the G and P genotypes detected and high prevalence G2P6 strains in the region with short profiles [15]

The segmented nature of the rotavirus genome and high rate of mutation [19] suggest that during a mixed infection with different rotavirus strains genetic reassortment (substitution of RNA segments between different RNA strands) could occur. Mosaic strains occurred mainly

163 in hospitalized children implying that rotavirus diarrhea could be more severe in West and
164 central African children where such strains are common [7,13,15].

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166 Unlike with many studies in the region that report high levels of untypeable strains [7, 13,
167 18], most of our isolates could be characterized by RT-PCR genotyping methods. Failure to
168 type rotavirus strains could result from several factors including presence of rare genotypes,
169 insufficient viral RNA genome, destruction of viral genome during repeated freeze thawing,
170 or due to mutations at specific binding sites in the target genome or primers [18-20]. Strains
171 that do not react with RT-PCR genotyping primers could be analyzed by a modification of
172 standard procedures, virus culture and sequencing [18, 20] that were beyond the scope of
173 our study.
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176 **Fig.3. Frequency (%) Distribution of rotavirus G/P combinations in North West**
177 **Cameroon (2004).**

178 *Gnt = Non-typeable VP7 genotype; Pnt = Non-typeable VP4 genotype*
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180 3.5 Age (months) Distribution of G/P combinations

181 The predominant G/P combinations in different age groups were: 0-3, G2P[6]; 4-6, G3P[8];
182 7- 9, G1P[8], 10-12: G2P[6]P[8]; 13-18 G3P[6]; 19-24 (G1P[8]; and 25-60, (G1P[6]) with
183 ages 7- 12 showing the highest strain diversity (Table 2). All mixed genotypes occurred in
184 children under 24 months of age while genotype G3P[6] (20%) was the most common strain
185 in hospitalize children. Genotype G1 P[8] is usually the most common strain detected in
186 most epidemiologic studies [6, 7, 13, 17]. Occurrence of G3P[6] (17.9%) as the second

187 most common strain could pose a problem in vaccine effectiveness if the current vaccines
188 do not produce sufficient heterotypic antibody protection against globally less common
189 strains such as P[6] if they become prominent in any region.

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191 **Table 2. Age distribution of rotavirus Genotypes combinations in North West**
192 **Cameroon (2004).**
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Age Group (months)								
Genotypes	0-3	4- 6	7-9	10-12	13-18	19-24	25-60	Total
G1P[6]	0	0	0	1	1	0	1	3
G1P[8]	0	0	6	1	2	2	0	11
G2P[6]	1	0	0	0	0	0	0	1
G2 P[8]	0	0	1	1	1	1	0	4
G3P[6]	0	0	4	1	3	2	0	10
G3P[8]	0	2	2	2	1	0	0	7
G1P[6]P[8]	0	0	0	1	2	0	0	3
G2P[6]P[8]	1	0	2	3	0	1	0	7
G9P[6]P[8]	0	0	1	0	0	1	0	2
G2G3P[6]	0	0	0	0	1	0	0	1
G2G9[6]	0	1	0	0	0	0	0	1
G1G3G8P[6]P[8]	0	0	0	0	1	0	0	1
G3Pnt	0	0	1	1	0	0	0	2
G1G3Pnt	0	0	0	0	1	0	0	1
Gnt P[6]	0	0	0	0	0	0	1	1
Gnt P[8]	0	0	0	0	0	1	0	1
Total	2	3	17	11	13	8	2	56

194 *Gnt = Non-typeable VP7 genotype; Pnt = Non-typeable VP4 genotype*

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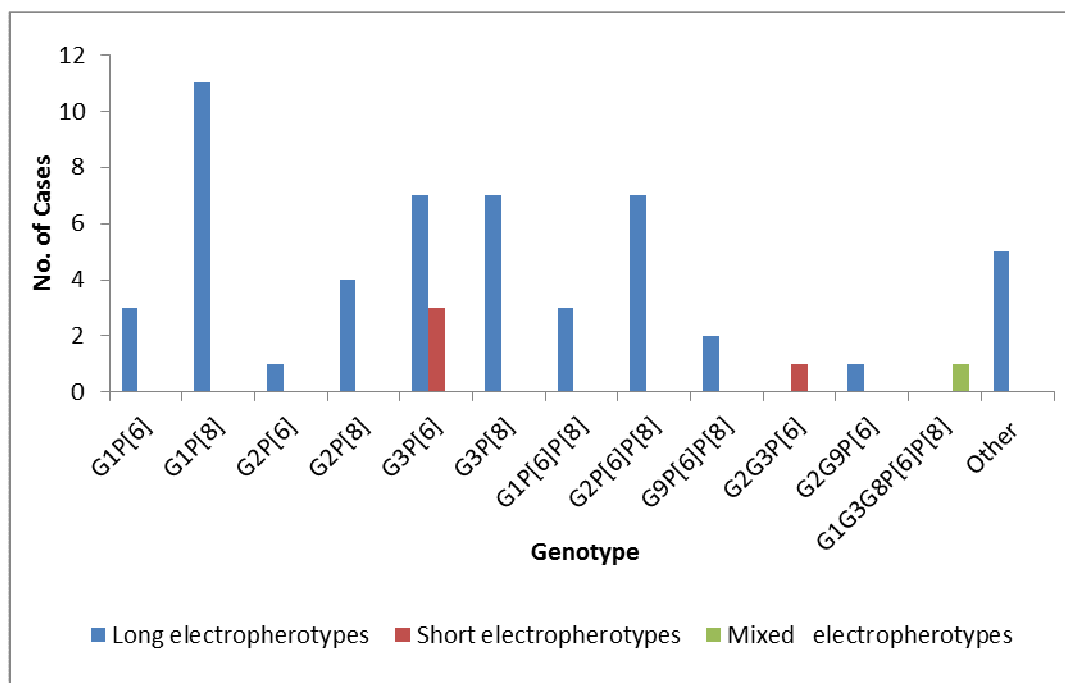
196 3.5. Genotype/electropherotype combination analysis

Several strains showed unusual genotypes/electropherotype combinations: all G2 and partially characterized strains had long electropherotypes while G3 strains showed both long and short electropherotype profiles (Figure 4). Strains with unusual genotype/electropherotype combinations (G2P[6], G2P[8], G3 P[6], G2P[6]P[8], G2G9P[6], G9P[6]P[8], G1G3P[6]) accounted for 20/56 (35.7%) of cases. All mixed G types occurred in hospitalized children. Rotavirus genotypes G1 and G3 typically have long electropherotypes and pair with P[8] while G2, G8 and G9 have short electropherotype profiles and pair with P[4]. Genotype G1 P[8] is usually the most abundant strain in circulation [5, 13]. Abnormal genotype-electropherotype and G/P combinations indicated high diversity of rotavirus strains both at genomic and molecular levels. However, some strains such as G3P[6] that showed short electropherotypes profiles might have been strains of G9 misidentified as G3 [19].

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G9 strains may also combine with P[6] [5] but strains presented with long RNA profiles and mixed VP4 specificity of P[6]P[8]. Three types of G9 strains have been reported based on electropherotype profile, VP7 and VP4 specificities in which strains either have long or short electropherotypes reacting with either P[4], P[6] or P[8] genotype specific primers [5]. This raises concerns about the possibility of a complex genetic pleomorphism among rotavirus strains that remain to be uncovered and questions about the specificity of genotyping primers for G9 strains that have been misidentification as G3 [19]. The detection of all G9 strains, mixed G types and those with atypical G/P - electropherotype combinations in hospitalized children further suggests the possibility of these strains being more virulent although host and environmental factors could also play a role in disease severity.

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Fig. 4. Rotavirus genotype and electropherotype combinations in North West Cameroon (2004)

4. CONCLUSION

This report presents the diversity of rotavirus G and P types circulating in the NWR of Cameroon in which genotypes G1-3 constituted over 85% of strains with predominance of G3P[6] in hospitalized children. G3P[6] emerged as the second most common strain after G1P[8]. Unusual strains and mixed infections circulated at high levels including P[6] strains detected in all age groups. These results provide the background for future evaluation of vaccine efficacy and strain evolution in Cameroon and the Central Africa region. Continuous strain surveillance is necessary for a better understanding of rotavirus strain diversity and the epidemiologic significance of G3, G8, G9, P[6] and unusual strains. Follow up studies intend to investigate post vaccination strain prevalence. Further research is also needed to confirm the association of mixed infections and unusual strains with disease severity that may elucidate the high rotavirus disease burden in Sub-Saharan Africa [1] and to clearly identify partially characterized strains and those with atypical genotypes or genotype/electropherotype combinations.

CONSENT

Informed consent was obtained from parents or guardians of all individuals who participated in the study

ETHICAL APPROVAL

This study was approved by the North West Regional Delegation of Public Health, Cameroon.

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