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Spatio-Temporal Dynamics of Circulating Rotavirus G1 Isolates Recovered from Children with Acute Flaccid Paralysis in Cameroon

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Abstract

Rotavirus (RV) is a virus that primarily affects the gastrointestinal tract of animals and humans. However, its extra-intestinal spread to the brain can lead to acute flaccid paralysis (AFP), a syndrome characterized by sudden weakness in one or more limbs. In Cameroon, the available data on RV come from cases of gastroenteritis and diarrhea. In children with AFP, there are no data on their genetic diversity and circulation. This study aims to determine the frequency of detection, genetic diversity, and circulation dynamics of RV in these children. A retrospective cross-sectional study was conducted among children aged 15 years or younger with AFP admitted to Cameroonian health facilities. RV detection was performed by real-time RT-PCR, genetic diversity by genotyping performed by Sanger sequencing of the VP7 and VP4 genes, and investigation of circulation dynamics through phylogeography. Data analysis and Pearson's chi-square test were performed using Excel and SPSS version 29 software. Phy-

logenetic analysis, supported by 1000 bootstrap replications, was performed using MEGA version 11 software. A p-value less than or equal to 0.05 was considered statistically significant. RVA was detected in 38% of cases (76/200), with genotype G1 (82.1%), P[8] 3.6% and G/P combination of G1P[8] (14.3%). Genetic distance analysis of RVA G1 strains has revealed the circulation of slightly similar strains in some Regions of Cameroon, suggesting the emergence of RVA epidemics in 2018. Phylogenetic analyses determined an evolutionary rate of 9.13×10^{-4} (HDP 95%: 4.5×10^{-4} - 2.8×10^{-3}) substitutions/site/year of RVA G1. Spatio-temporal circulation analysis revealed that Maputo in Mozambique was the country of origin of the Cameroonian strains, which are believed to have migrated to Cameroon and then spread to other Cameroon Regions, such as the Far North from the Littoral Region onwards, facilitated by human migration. Our results highlight that a silent RVA G1 epidemic circulated in Cameroon in 2018, providing valuable data for the surveillance and management of the emergence of RVA G1 strains not targeted by the vaccination in Cameroon.

Keywords

Rotavirus, Acute Flaccid Paralysis, Phytogeography, Cameroon

1. Introduction

Rotavirus (RV) was first discovered in 1973 from stool samples from children with severe diarrhea [1]. RVs are non-enveloped viruses with a viral particle diameter of 70 nm and 100 nm. Their icosahedral capsid is made up of a triple layer of proteins: the outer, middle, and inner (or core) layers. It contains the enzymes necessary for viral replication, including an RNA-dependent RNA polymerase. The surface of the capsid (outer layer) consists of the viral protein 7 (VP7) glycoprotein surmounted by spicules formed by the VP4 protein [2]. The RV genome is made up of 11 segments of double-stranded RNA, which code for 6 structural proteins (VP1, VP2, VP3, VP4, VP6, and VP7) and 6 non-structural proteins (NSP1 to 6). Each segment encodes a structural protein, except segment 11, which encodes the NSP5 and NSP6 proteins [3]. The VP4 and VP7 proteins make it possible to classify RV into genotype P and G, so there are 51 and 36, respectively [4].

RVs belong to the family *Reoviridae*, genus *Rotavirus*. The genus *Rotavirus* contains 10 recognized virus groups: *Rotavirus* A, B, C, D, E, F, G, H, I, and J. Four groups of RVs (A, B, C, and H) are described as etiologic agents of diseases in humans [5]. Group A is the most commonly identified and consists of 37 genotype G and 51 genotype [6]. In contrast to RV group B to J, which cause gastrointestinal symptoms as well as severe diseases, such as RVA, which was associated with neurological damage: encephalitis, cerebellitis, encephalocerebellitis, and acute flaccid paralysis (AFP) [7] [8]. AFP is defined as weakness of a limb in a patient [9]. AFP surveillance consists of the virological investigation of flaccid paralysis in children < 15 years with suspected poliomyelitis cases. Once attributed solely to

enteroviruses, AFP is now known to stem from diverse infectious and non-infectious causes, including trauma, toxins, and metabolic disorders [10]. Apart from enteroviruses, other enteric viruses such as RVs that colonize the gastrointestinal tract may disseminate to secondary organs (central nervous system where they can trigger acute flaccid paralysis (AFP) [7].

Rotavirus (RV) is transmitted by the direct oral faecal route from person to person or indirectly through the consumption of contaminated food or drink [1]. Several factors thus facilitate their propagation dynamics during epidemics: the degree of recombination of their genomes, taxonomic classification, genome size, type and segmentation, the absence of an outer envelope, the duration of infection, and host mortality [11]. Phylogeography also exploits the probability of transmission of strains between Regions and countries in relation to time in order to define the circulation dynamics of different genotypes [12]. Although the rotavirus (RV) involvement in neurological diseases remains difficult to exclude, studies conducted in Italy, Gabon, and Nigeria have reported the detection of RVs among acute flaccid paralysis (AFP) cases [13]-[16]. Despite the use of vaccines to control rotavirus infections, they continue to cause significant morbidity and mortality [8]. Between 2013 and 2017, the annual number of child deaths due to rotavirus was between 122,000 and 215,000 [17]. In most low-income countries in Asia and Africa, the epidemiology of rotavirus is characterised by one or more periods of relatively intense circulation of the virus during the year, compared with high-income countries, which experience transmission during the winter [18]. The genotypes most commonly found worldwide are G1, G2, G3, G4, and G9. These genotypes represent around 90% of the strains genotyped worldwide [19]. Similarly, for Rotaviruses belonging to the P genotypes, the most frequent are P[4] and P[8], and rarely the genotypes P[6] or P[9], P[3], P[11], P[14], and P[12]. Some of these genotypes are the result of the transmission to humans of animal Rotaviruses (bovine, porcine), which are believed to have been passed on to humans and to be at the origin of human-animal reassortments [20]. Nowadays, we are seeing the emergence of Rotavirus co-infections belonging to several G/P genotypic combinations. The main types found are G1P1 to [8], G2P1, G3P1 to [8], G4P1 to [8]; their relative appearance changes over time from one place to another [3]. Geographical and temporal fluctuations have been noted in the distribution of different Rotavirus (RV) strains depending on the years and localities studied; new Rotavirus genotypes such as RVA G9 have been studied and their transmission dynamics described [20]. This is the case in France, where heterogeneous circulations of RV strains have been noted depending on the season and locality [21].

In Cameroon, although Rotarix vaccination was introduced into the Expanded Programme on Immunisation (EPI) in 2014 to limit the incidence of rotavirus infections in children, vaccination coverage against the rotavirus is still very low, and children continue to die [22]. The majority of published studies look for RV in the stools of children with gastroenteritis and diarrhea. A recent meta-analysis study carried out on this subject by Njifon and colleagues determined a prevalence

of 29.8 % [23]. RVs have rarely been considered in acute paralysis cases. There is no data on RV prevalence even less on the circulation of these viruses in children suffering from acute flaccid paralysis in Cameroon. This study aimed to determine the detection frequency, genetic diversity, and circulation dynamics of rotaviruses circulating among children under 15 years of age with acute flaccid paralysis in Cameroon.

2. Materials and Methods

2.1. Study Design

This study was a retrospective study carried out on a biological collection of 200 stool specimens collected in children with acute flaccid paralysis (AFP) cases from January 01, 2018, to December 31, 2019. AFP cases were notified in children ≤ 15 years received in any Cameroon health facility. Stool samples were collected following medical consultation and transported under reverse cold-chain conditions (2˚C - 8 ˚C) to the WHO-accredited Intercountry Reference Laboratory for poliomyelitis Centre Pasteur du Cameroun (CPC). Cameroon exhibits in its Regions remarkable climatic and agroecological diversity. The savannah and steppe landscapes characterized the northern areas of Cameroon and experience a short rainy season from April to July. The rest of the country is under an equatorial climate and is characterized by two rainy seasons (March-June and September-November) and two dry seasons. The South, East, and Centre Regions are dominated by dense equatorial rainforests. The West and North-West feature g grasslands, highlands and montane forests. The South-West and Littoral Regions form the coastal belt in direct contact with the Atlantic Ocean [24].

2.2. Selection of Samples

Upon receipt of stool samples at the Centre Pasteur du Cameroun (CPC), enterovirus isolation was carried out in cell culture following WHO-recommended protocols [25]-[27]. A total of 200 stool samples were randomly selected, stratified by month of onset and region, from the collection of samples negative for enteroviruses by viral isolation on Human rhabdomyosarcoma (RD), human larynx epidermoid carcinoma (HEp-2c), and murine L20B (a derivative of murine L cells expressing the poliovirus human receptor) cell cultures from children with AFP between 2018 and 2019. The number of samples was determined using the Lorentz formula, assuming a prevalence of EV of 16% [28], and the final selection was made by reasoned sampling using R software from the 3669 negative samples listed in the laboratory's Epi Info database. The selected samples were then sorted and stored at −20˚C until the extraction stage.

2.3. Nucleic Acids Extraction and Detection of Rotavirus RNA by Real-Time RT-PCR

Rotavirus (RV) RNA was extracted from 20% (weight/volume) of stool sample suspensions using the Quick-RNA Viral Kit (ZYMO Research) per the manufac-

turer's protocol [29]. Purified RV RNA was eluted in 50 µL RNase/DNase water and stored at −80˚C until they were tested for RV RNA by rRT-PCR. Molecular detection of RV RNA was performed on Biorad CFX 96 (Real-Time PCR Detection System—Gene) as previously described [1] using the set of primers and probe described by Freeman in 2008 [30]. A positive result was defined by a sigmoidal amplification curve and a CT < 37 for stool samples.

2.4. RT PCR, Sequencing, and Phylogenetic Analyses of Rotavirus

Conventional PCR was performed on all RNA samples with CT < 37 after Real-Time PCR to amplify a portion of 881 and 512 nucleotide portions of the VP4 and VP7 coding gene of rotaviruses (RV) to determine the G genotypes using the VP7-F and VP7-R primers, and genotype P using the VP4-F and VP4-R primers as previously described. PCR was performed according to the protocol described by Gouvea, Iturriza, and Gray, respectively [31] [32]. Gel green® (Invitrogen, Carlsbad, CA, USA) stained agarose gels were used to analyze five microliters of PCR results, which were then exposed to a UV transilluminator.

The QIAquick PCR purification kit (Qiagen, Courtaboeuf, France) was used to purify the resulting amplicons. This was followed by direct sequencing of both strands using nested PCR primers and the Big Dye terminator v3.1 kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3140 automated sequencer. Multiple sequence alignment, consensus, and contig sequences were produced using the CLC Main Workbench 21.0.3 programs (Qiagen, France). Under the nucleotide sequence accession numbers PP551485 to PP551507, PP578957, and PP578953 to PP578956, newly discovered sequences were added to the GenBank database. MEGA version 6.0 software was used to perform Maximum Likelihood phylogenetic analysis using the best-fit GTR+G+I+4 model. A bootstrap pseudo-replicate of 1000 trees was used to estimate the trustworthiness of each tree topology.

2.5. Calculation of Genetic Distances of Rotavirus

The evolutionary distances between the Rotavirus (RV) sequences obtained in this study were estimated by calculating the proportion of nucleotide differences between each pair of sequences to be compared using MEGA software [33]. The evolutionary distances between the RV sequences obtained during this study were estimated by calculating the proportion of inter- and intra-nucleotide differences between the study sequences and the reference sequences using MEGA software when the genetic distance (GD) = 0, the sequences were closely related, derived from a recent common ancestor, or were the same strain; the mode of circulation was epidemic. When GD > 0, the sequences were all different, and the mode of spread was endemic. If 0 < GD ≤ 0.2, the strains compared belonged to the same clade and to different clades for GD > 0.2 [34]. The average percentage of similarity was deduced by [1 − GD] × 100. The precise estimate of genetic distance was made by calculating the standard error (SE). When SE < 0.01, we had an accurate

estimate of GD. SE > 0.05, high uncertainty of GD results (interpreted with caution) [35].

2.6. Phylogeography of Rotavirus Strains Genotyped

To analyse the circulation dynamics (phylogeography) of enteric viruses in Cameroon, a phylogeographic analysis was carried out on sequenced samples using the bioinformatics software Nextstrain [36]. All study sequences and reference sequences used to construct phylogenetic trees were imported into Nextstrain software, then indexed and aligned using the "augur index and align" commands. Then, the pipeline thus constructed progressed, and the maximum credibility phylogenetic tree of the clades containing the study sequences and the reference sequence most identical to those of the study was constructed by clicking on "augur tree", incorporating temporal information through the use of a time tree and a clock rate defined during the augur refine refinement step. Parameters such as a clock rate of 0.0007 and an estimated reading time of less than 2 minutes were recorded.

A separate customised step, "prune_outgroup", using "nw_prune" [37], is then deployed to selectively choose the reference sequence used to root the tree, ensuring that attention is focused on the evolutionary relationships between the sampled sequences.

The steps "ancestral augur", "translate", and "trait", which aim to improve the accuracy of the maximum credibility phylogenetic tree, were selected. The next step was to select "inference" to enrich the tree by providing estimates of the dates of occurrence of evolutionary events. The "translate" and "trait" options were then used. Visualisation was performed using the complementary website Auspice (auspice.us), thanks to the JSON export file generated at the end of the Nextstrain run [36].

2.7. Data Analysis

All Statistical analyses used in this study were performed using Microsoft Excel (Microsoft Corp., Washington, DC, USA). Anonymized epidemiological patients' data were registered with laboratory results, then verified and cleaned to correct any errors in entries prior to analysis. The analytical focus was on prevalence and seasonal trends. The chi-square (χ^2) test was conducted to compare prevalence rates. The statistical significance was defined for a P-value below 0.05. Values above this threshold were considered non-significant. The CLC Mainwork bench software was used for phylogenetic analyses and genetic distance calculations, and Nextstrain was used for phylogenetic analyses.

3. Results

3.1. Demographic Data of the AFP Cases Analyzed

A total of 200 stool samples were randomly selected following stratification based on region of origin and month of disease onset. Among 1552 eligible AFP cases received in the Centre Pasteur du Cameroun laboratory between January 2018 and

December 2019, these selected cases, all aged ≤ 15 years, provided at most two stool specimens each. Of the sampled children, 146 had ages ranging from 3 months to 15 years, with a mean age of 4.64 ± 1.16 years (Table 1).

Table 1. Demographic, epidemiological, and virological characteristics of the study population of acute flaccid paralysis children originating from Cameroon from 2018 to 2019.

Characteristics	Rotavirus			
	Total	Negative	Positive	P-value
	No (200)	124 (62%)	76 (38%)	
Gender				
Male	125 (62.5%)	79 (63.7%)	46 (60.5%)	
Female	75 (37.5%)	45 (36.3%)	30 (39.5%)	0.4
Median [IQR]	4.6 [4.25 - 5.03]	3 [2.5 - 3.5]	4.75 [3.57 - 5.93]	
Age				
0 - 2 y	26 (13%)	14 (11.3%)	12 (15. 8%)	
2 - 5 y	**65 (32.5%)**	**41 (33.1%)**	**24 (31.6%)**	0.21
5 - 15 y	55 (27.5%)	35 (28.2%)	20 (26.3%)	
Missing	54 (27%)	34 (27.4%)	20 (26.3%)	
Year				
2018	109 (54.5)	59 (47.6%)	50 (65.8)	0.028
2019	91 (45.5)	65 (52.4%)	26 (34.2)	

Note: Confidence interval at 5% alpha risk of being wrong; Children with missing age information are designated as "Missing"; P values ≤ 0.05 were considered statistically significant for comparisons of the respective variables.

Age distribution was as follows: 26 children (17.8%) were under 2 years old, 65 (44.5%) were aged 2 to 5 years, and 55 (37.7%) fell into the 5 to 15 year range. The cohort comprised 125 males (62.5%) and 75 females (37.5%).

3.2. Rate of Rotavirus Detection in Children with AFP

The characteristics of sex, age, and the rate of RV detection were statistically comparable during the 2-year study period (Table 1). Overall, 38% (76/200) of the studied samples were tested positive for RV RNA (Table 1). The most affected age group was 2 to 5 years, thus suggesting that children and infants may be at greater risk of RV infection [38]-[41]. Age data were missing for 54 AFP cases and were thus excluded from the analysis of the association between age and RV detection. The rate of RV RNA detection was only significantly associated with year of detection ($P < 0.05$). The mean age of RV-positive children was 4.67 years (Table 1). The proportion of RV-positive children was highest in children between 2 and 5 years of age (24/76, 31.6%) and decreased in the other age ranges.

3.3. Distribution of Rotaviruses by Study Region in Cameroon

By studying the distribution of enteritis viruses in the Regions of Cameroon, Rotavirus A (RVA) appears to have circulated in all Regions. The Far North Region (15/76, 19.7%) and the Littoral Region (10/76, 13.1%) were the most conducive to the study of RVA. For the Central Region, where several studies have been conducted, the prevalence of RVA obtained during this study was 10.5% (Table 2).

Table 2. Distribution of rotavirus strains detected by rRT-PCR and genotyped by SANGER sequencing by study region in Cameroon from 2018 to 2019.

Regions		rRT-PCR		Phylogenetic Analysis (Genotypes)			
	Total	Negative	Positive	Total	G1	P8	G1P8
	No (200)	124 (62%)	76 (38%)	28	23 (82.1%)	1(3.6%)	4 (14.3%)
Adamaoua	22 (11%)	11(8.9%)	11 (14.5%)		4 (17.4%)		
Center	22 (11%)	14 (11.3%)	8 (10.5%)			1 (100%)	1 (25%)
East	21 (10.5%)	14 (11.3%)	7 (9.2%)		3 (13.04%)		
Far North	38 (19%)	23 (18.5)	15 (19.7%)		4 (17.4%)		3 (75%)
Littoral	20 (10%)	10 (8.06%)	10 (13.1%)		5 (21.7%)		
North	23 (11.5%)	14 (11.3%)	9 (11.8%)		3 (13.04%)		
North-West	9 (4.5%)	5 (4.03%)	4 (5.2%)		2 (8.7%)		
West	24 (12%)	15(12.09%)	9 (11.8%)		2 (8.7%)		
South	17 (8.5%)	15 (12.09%)	2 (2.6%)				
South-West	4 (2%)	3 (2.41%)	1 (1.3%)				

3.4. Temporal Distribution of Rotavirus Detected in Childreen with AFP Patients

By studying the seasonality of Rotavirus A (RVA), it appears that these viruses circulated in Cameroon throughout the study period, regardless of their origin and month of collection during all months of the study period (Figure 1). RVA detection peaked during the months of March and April, suggesting intense circulation of these viruses during the rainy seasons.

3.5. Genotype Assignment and Phylogenetic Relationships of Detected Rotavirus

Overall, 76 RVA-positive samples with CT values below or equal to 37 were available for conventional RT-PCR, yielding an amplification success rate of 47.4% (36/76). Among these, 26 samples (34.2%) amplified only the VP7 gene, 4 samples (5.2%) only the VP4 gene, and 6 samples (7.9%) for both VP7 and VP4 genes. The VP4 and VP7 regions of all 36 amplified samples were successfully sequenced. Of these, 28 (77.8%) produced exploitable sequences, while the remaining exhibited uninterpretable electropherograms with overlapping peaks. BLAST analyses performed on the NCBI platform allow the selection of nucleotide sequences with

identities of ≥97% with the sequences of this study, confirming homology with reference homotypic sequences from GenBank. Combined with pairwise comparisons, phylogenetic analyses enabled genotype assignments for the newly sequenced RVAs, with 82.1% (23/28) classified as G1, and deposited in GenBank under the following accession numbers: PP551485 to PP551507. These RVA GI sequences were grouped with homologous strains from different geographical Regions of the world, in particular with RVA strains that circulated in Mozambique (KP22808.1), Benin (MZ065850), India (KT387241.1; KT387243.1), Angola (KT225642.1), and Cameroon (KM660406.1, KM660407.1) (Figure 2).

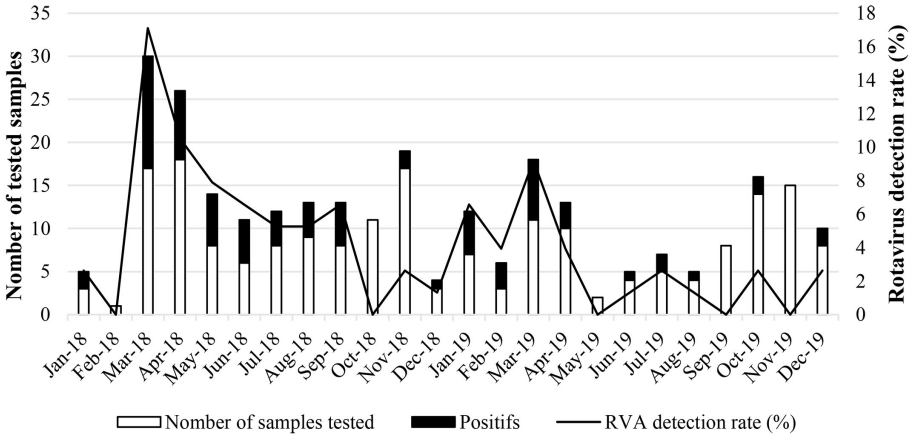

Figure 1. Monthly distribution of Rotaviruses in children with acute flaccid paralysis in Cameroon from January 2018 to December 2019. The primary left y-axis and bars describe the number of stool specimens tested, whereas the secondary right y-axis and lines describe the monthly RVA detection rate.

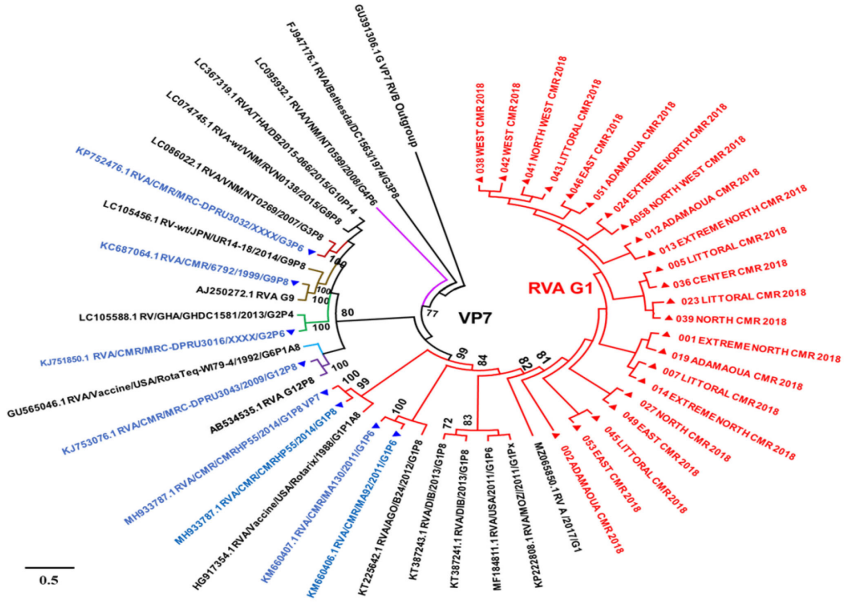

Figure 2. Maximum-likelihood phylogenetic tree of the partial VP7 coding gene depicting the phylogenetic relationships of the RVAs from Cameroon.

The phylogenetic tree was estimated from an 881-nucleotide (nt) sequence alignment using the maximum-likelihood (ML) method with the best-fit nucleotide substitution model (GTR+G+I+4) determined by Smart Model Selection implemented in the PhyML software. Newly sequenced RVAs are indicated by a red triangle (▲) while previously reported sequences from Cameroon are indicated by a blue triangle (▲). Sequences originating from Cameroon are labelled according to the country with the respective laboratory serial number, region of origin, country code CMR, and year of sampling. Country names have been abbreviated according to the ISO 3166-1 standard: ANG, Angola; DIB, Dibrugarh; GHA, Ghana; JPN, Japan; MOZ, Mozambique; THA, Thailand; USA, United States of America, and VNM, Vietnam. The GenBank accession number, genotype, location, and year of sampling of reference sequences are indicated on the tree. The rotavirus B (Acc. No. GU391306.1) was used as an outgroup for the orientation of the tree. For clarity, most bootstrap values below 70% have been omitted. Scale bars indicate the nucleotide distance as substitutions per site.

Of the four RVA amplified by conventional PCR genotyping, only one virus (25%) amplified only the VP4 gene of RVA and was sequenced. This unique VP4 nucleotide sequence has been deposited in GenBank under the following accession number: PP578957. Phylogenetic analyses of this unique sequence of the VP4 gene of group A rotaviruses (RVA), branching into the group of other P8 genotype RVA sequences that were reported in Cameroon in 2014 and in other countries, like the United States of America in 2009 (Figure 3).

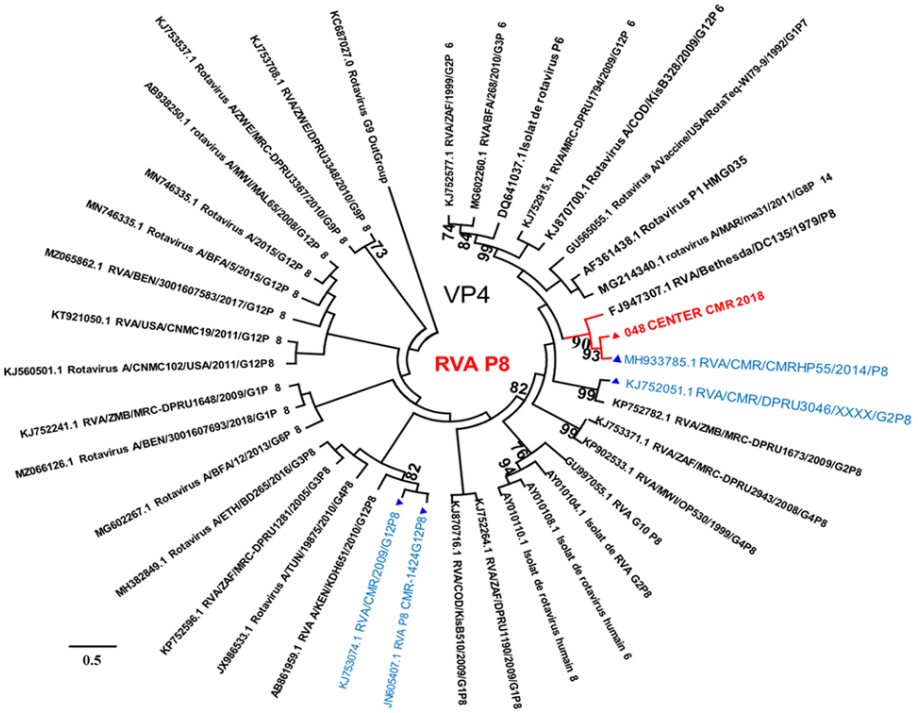

Figure 3. A maximum-likelihood phylogenetic tree was constructed to illustrate the genetic relationships among Cameroonian RVA strains based on partial VP4 gene sequences.

The analysis was performed on a 512-nucleotide alignment using the ML method, applying the optimal substitution model (GTR+G+I+4) as identified by Smart Model Selection within the PhyML software. Newly sequenced RVAs are indicated by a red triangle (▲) while previously reported sequences from Cameroon are indicated by a blue triangle (▲). Sequences originating from Cameroon are labelled according to the country with the respective laboratory serial number, region of origin, country code CMR, and year of sampling. Country names have been abbreviated according to the ISO 3166-1 standard: BEN, Benin; BFA, Burkina Faso; COD, Democratic Republic of Congo; ETH, Ethiopia; KEN, Kenya; MAR, Morocco; MRC, Mauritania; MWI, Malawi; TUN, Tunisia; USA, United States; ZAF, South Africa; ZMB, Zambia and ZWE, Zimbabwe. The GenBank accession number, genotype, location, and year of sampling of reference sequences are indicated on the tree. The rotavirus B (Acc. No. KC687027.0) was used as an outgroup for the orientation of the tree. For clarity, most bootstrap values below 70% have been omitted. Scale bars indicate the nucleotide distance as substitutions per site.

The third series of sequences represents 14.3% (4/28). Rotavirus A (RVA) RNA amplified simultaneously with the VP4 and VP7 genes was detected in 6 of 76 (7.9%) stool samples from children with acute flaccid paralysis and analyzed by endpoint RT-PCR. After sequencing, four samples had usable sequences. Phylogenetic analyses showed that they all belonged to the G1P8 genotypic combination. The nucleotide sequences were deposited in GenBank with the following accession numbers: PP578953 to PP578956. The G1P8 RVA sequences showed close genetic relatedness to an RVA strain MZ065850.1 originating in Benin in 2017 (Figure 4).

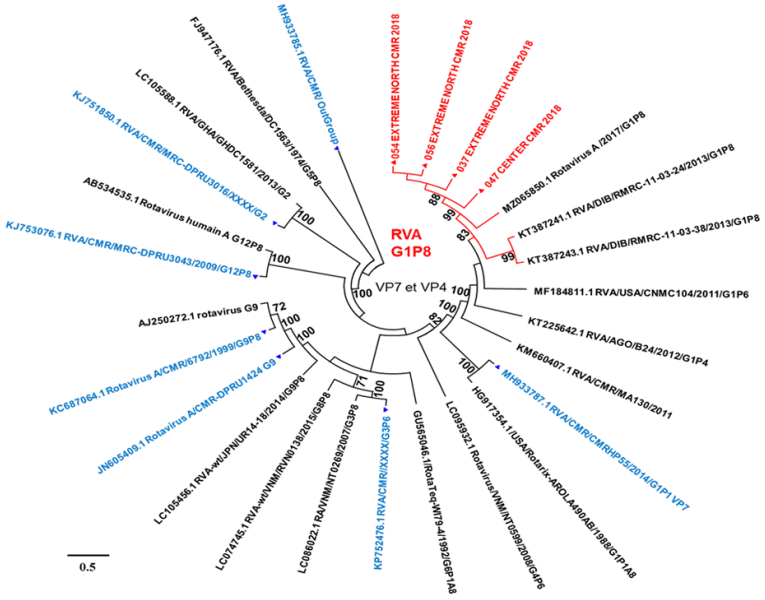

Figure 4. A maximum-likelihood phylogenetic tree was constructed to illustrate the genetic relationships among Cameroonian RVA strains based on partial VP4 and VP7 gene sequences.

The analysis was performed on a 512 - 881 nucleotide alignment using the ML method, applying the optimal substitution model (GTR + G + I + 4) as identified by Smart Model Selection within the PhyML software. Newly sequenced RVAs are indicated by a red triangle (▲) while previously reported sequences from Cameroon are indicated by a blue triangle (▲). Sequences originating from Cameroon are labelled according to the country with the respective laboratory serial number, region of origin, country code CMR, and year of sampling. Country names have been abbreviated according to the ISO 3166-1 standard: ANG, Angola; DIB, Dibrugarh; GHA, Ghana; JPN, Japan; USA, United States of America, and VNM, Viêt Nam. The GenBank accession number, genotype, location, and year of sampling of reference sequences are indicated on the tree. The Rotavirus A [P8] (Acc. No. MH933785.1) was used as an outgroup for the orientation of the tree. For clarity, most bootstrap values below 70% have been omitted. Scale bars indicate the nucleotide distance as substitutions per site.

3.6. Calculation of Genetic Distances of Rotavirus

3.6.1. Distribution of Genotyped G1 RVAs by Region in Cameroon during the Study Period

Rotavirus A genotype G1 (RVA G1) sequences were obtained from 7 of Cameroon's 10 regions during 2018. The Littoral Region alone accounted for 21.7% (5/23) of the sequenced RVAs (Table 2).

3.6.2. Estimation of Average Genetic Distances between the G1 Sequences in the Study and the Reference Sequences

A total of 23 Rotavirus A (RVA) G1 sequences analyzed in this study were compared among themselves and against seven reference strains (RVA G1, G9, and P8 genotype as outgroup). The average genetic distance between the studied sequences and G1 references was 0.03 (3% divergence) with a standard error of 0.006, whereas this level of similarity was not observed with other reference genotypes (Table 3).

Table 3. Estimation of the average genetic distance between Rotavirus G1 study sequences and reference sequences by region and year of study in Cameroon.

	RVA G1 (4)	RVA G9 (2)	RV Out Group (1)	Study Sequences (23)
RVA G1 (4)	0.212 ES: 0.001			
RVA G9 (2)	0.256 ES: 0.014	0.254 SE: 0.014		
Rotavirus Out Group (1)	0.518 ES: 0.017	0.550 SE: 0.017	0.0 SE: 0.0	
Study Sequences (23)	**0.03** **ES: 0.006**	0.254 ES: 0.014	0.517 ES: 0.017	**0.012** **ES: 0.005**

Note: SE: standard error. The number of sequences for each RVA genotype and the sequences in the study are indicated in parentheses. Genetic distances were calculated between the reference genotypes and the sequences studied. Genetic distances are indicated in bold in each genotype and study sequence. In addition, average genetic distances are displayed with standard deviation estimates.

The intra-group genetic distance among the G1 sequences was lower, averaging 0.012 (1.2%) with an average standard error of 0.005, suggesting strong genetic relatedness and potential epidemic-level circulation. These RVA strains were detected in samples collected from various geographic locations and at different time points. Amplification controls and sequencing of 23 randomly selected positive stool specimens confirmed their origin from distinct individuals, ruling out any laboratory contamination. These findings point to a circulating strain potentially responsible for an undocumented rotavirus outbreak in Cameroon during 2018, which escaped detection by the national surveillance system.

3.6.3. Estimation of Average Inter- and Intra-Genotypic Genetic Distances between Rotavirus G1 Sequences from the Study and Reference Sequences by Study Region

For Rotavirus A genotype G1 (RVA G1) strains circulating in Cameroon in 2018, the average genetic distance between reference genotypes was 0.225 (SE: 0.011), corresponding to 22% divergence. In comparison, the average genetic distance between these references and the study sequences ranged from 0.136 to 0.142, with specific distances of 0.139 and 0.140 (SE: 0.008; 0.009) observed in the Far North, North, West, and East regions, representing an average divergence of 14%. Similarly, values of 0.141 (SE: 0.009) and 0.132 (SE: 0.008), indicating divergences of 14.1% and 13%, were observed for the Adamaoua and North-West regions, respectively. These results support the classification of all detected strains within the RVA G1 clade. Pairwise genetic distance analysis between sequences from different regions (e.g., Far North and North, Far North-West, Far North-North West, West-North, East-North, West-East, North West-North, East-West) consistently yielded values of 0 (SE: 0), suggesting genetic identity among strains and implying a single strain circulated across these locations during 2018. In contrast, intra-regional analyses revealed zero divergence within the North, West, East, and North-West regions, consistent with localized transmission of a single strain. However, non-zero distances observed in the Far North, Adamaoua, and Littoral regions suggest the co-circulation of multiple variants or distinct strains, with moderate genetic divergence noted in 2018 (Table 4).

Table 4. Estimation of average inter- and intra-genotype genetic distances between different Rotavirus A G1 sequences by region and year of study in Cameroon.

	Far North 2018 (4)	Adamaoua 2018 (4)	Littoral 2018 (5)	North 2018 (3)	West 2018 (2)	East 2018 (3)	North West 2018 (2)	Reference Sequences (7)
Far North 2018 (4)	**0.005** ES: 0.001							
Adamaoua 2018 (4)	**0.248** ES: 0.006	**0.203** ES: 0.004						
Littoral 2018 (5)	**0.244** ES: 0.005	**0.149** ES: 0.003	**0.094** ES: 0.002					
North 2018 (3)	**0** ES: 0	**0.149** ES: 0.003	**0.093** ES: 0.002	**0** ES: 0				

Continued

West 2018 (2)	**0** ES: 0	**0.148** ES:0.004	**0.082** ES: 0.002	**0** ES: 0	**0** 0			
East 2018 (3)	**0.005** ES: 0.001	**0.151** ES:0.003	**0.082** ES: 0.002	**0** ES: 0	**0** ES: 0	**0** 0		
North West 2018 (2)	**0** ES: 0	**0.149** ES: 0.004	**0.080** ES: 0.002	**0** ES: 0	**0** ES: 0	**0** ES: 0	**0** 0	
Reference Sequences (7)	**0.139** ES: 0.08	**0.141** ES: 0.009	**0.145** ES: 0.008	**0.14** ES: 0.009	**0.14** ES: 0.009	**0.0139** ES: 0.009	**0.132** ES: 0.008	**0.225** ES: 0.011

Note: SE: standard error. The number of sequences for each RVA genotype and the sequences in the study are indicated in parentheses. Genetic distances were calculated between the reference genotypes and the sequences studied. Genetic distances are indicated in bold in each genotype and study sequence. In addition, average genetic distances are displayed with standard deviation estimates.

3.7. Temporal Dynamics and Evolutionary History of Rotavirus A G1 Strains in Cameroon from 2018 to 2019

Linear regression analysis of the hypothetical root of genetic diversity of Rotavirus A (RVA) strains detected in Cameroon from 2018 to 2019 revealed a strong temporal signal from the analyzed dataset. This temporal signal showed a correlation coefficient of 0.7 for the relationship between root-to-tip divergence and the sampling date of the different sequences studied (**Figure 5**).

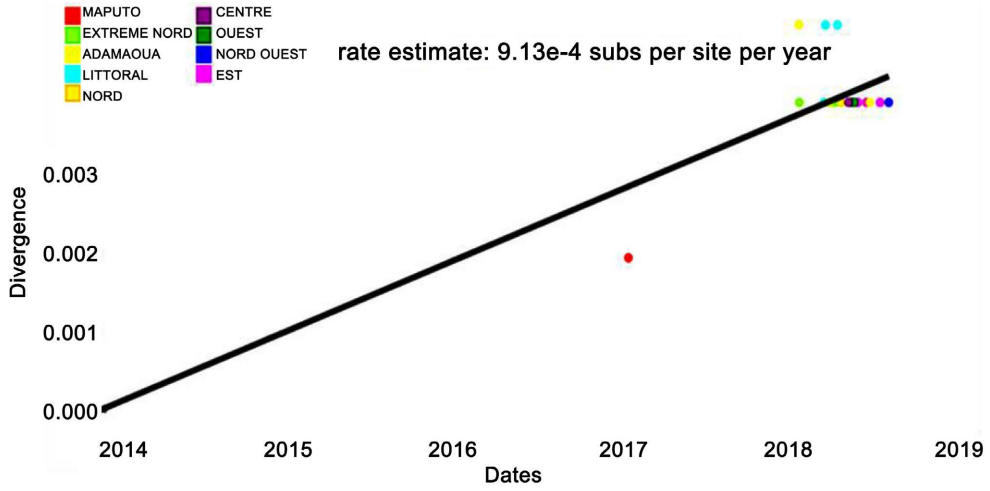

Figure 5. Regression of root-to-tip genetic distances relative to sampling dates for Rotavirus A detected in Cameroon from 2018 to 2019. The y-axis shows the differences noted, while the x-axis shows the sampling years and the points corresponding to the different samples.

The estimation of the temporal scale of the evolutionary history of the 43 Rotavirus A (RVA) sequences in the study and the reference sequences recorded worldwide in public reference databases such as NCBI (https://www.ncbi.nlm.nih.gov/), GISAID (https://gisaid.org/), and ViPR (https://www.viprbrc.org) was obtained using a Bayesian approach with Nextstrain software. The average nucleotide substitution rate in the RVA genome was 9.13×10^{-4} substitutions per site per year

(95% highest posterior density [HPD] interval, 4.5×10^{-4} - 2.8×10^{-3} substitutions/site/ year). The rate of substitutions per site per year for RVA obtained in this study falls within the standard range of values already described in the literature by other authors [42].

3.8. Spatial Propagation and Phylogeography of Rotavirus A Variants in Cameroon

The combination of spatial data with molecular data to study the dynamics of Rotavirus A (RVA) variant spread between different regions of Cameroon showed that RVA strains appeared in the city of Maputo in Mozambique and migrated to Cameroon, then spread to other regions of Cameroon such as the Far North, Adamaoua, North, Center, West, East, and Northwest (Figure 6).

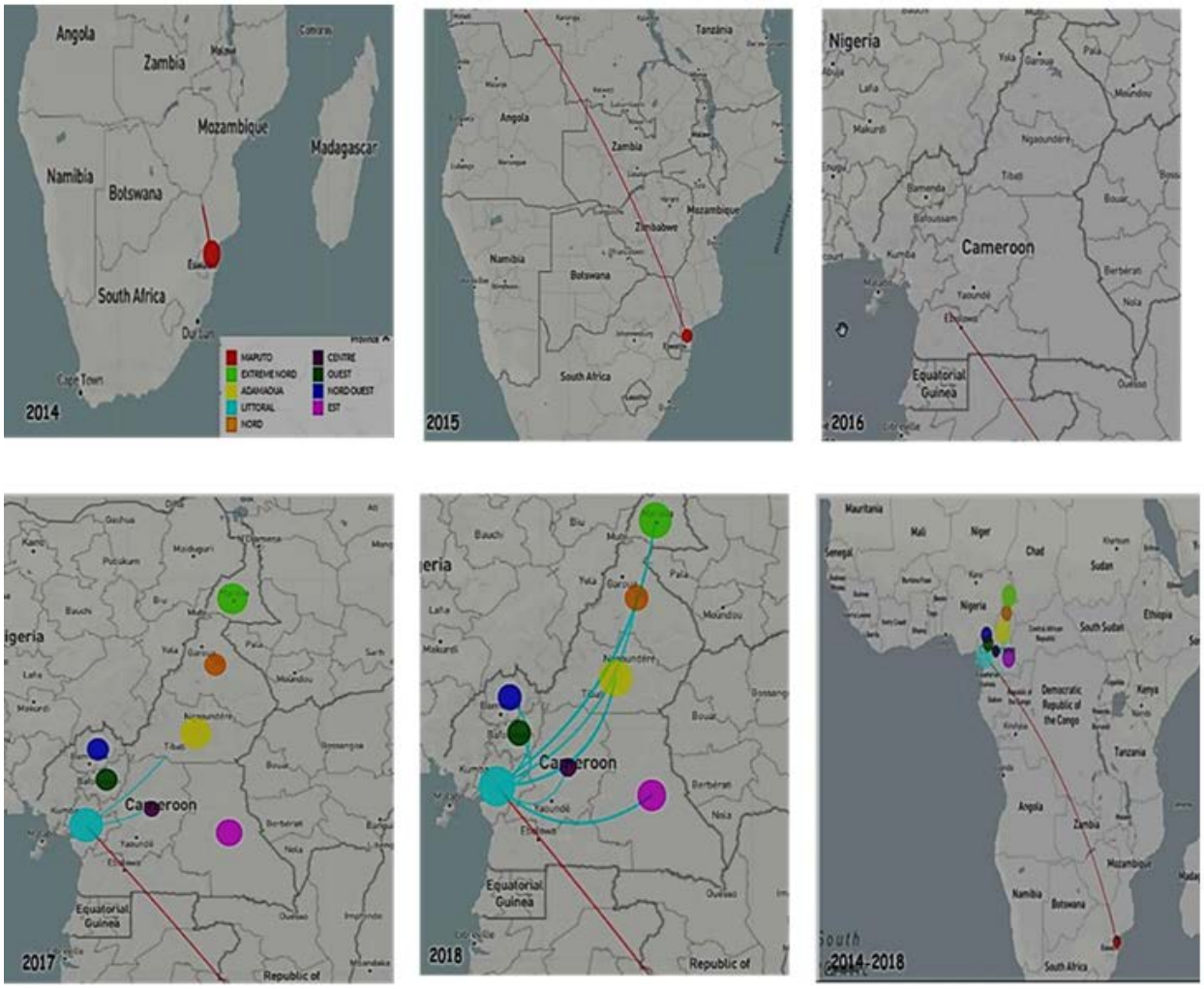

Figure 6. Geographical projection of the phylogenetic relationships of the RVA strains studied on a map illustrating the spatial dynamics of RVA circulation between the regions of Cameroon.

The genetic proximity of the Rotavirus strains characterized in this study suggests an epidemic of Rotavirus A (RVA). These enteric viruses appear to have per-

sisted in Cameroon only in 2018 through the continuous spread of these RVAs between Mozambique and the regions of Cameroon, as shown in **Figure 6**. The Littoral Region, the main source of infection, communicates with most of the neighboring regions (Center, West, Northwest), as well as more distant regions: Adamaoua, North, and Northwest. The spread of RVAs is thought to have occurred through the movement of people between countries before passing through urban and rural areas between Cameroon's regions. These waves of direct spread were amplified by the desire of people in the regions studied to travel there for visits or to stock up on supplies in Cameroon's leading economic city, thus acting as vehicles for the spread of these RVAs from one region to another.

4. Discussion

This study is the first to report both the detection rate and genetic diversity of rotavirus (RV) strains detected in children aged ≤ 15 years suffering from paralysis. Over the study period (January 01/ 2018, to December 31/2019), RVs were detected in 38.0% (76/200) of cases. The high circulation rate and genetic heterogeneity of RV strains observed in Cameroon may be due to their low environmental infectious dose, with 90% of viral particles remaining viable up to 60 days post-exposure and also because their transmission may also be linked to the consumption of water or beverages contaminated with RVs, which have been shown to persist in tap water for over 64 days at 20˚C, as previously documented by Trask (2012) and Greenburg *et al.* (2009) [43] [44]. The high frequency of RVA detection has already been reported in other studies, notably those on the epidemiology of enteric viruses in children under 5 years of age suffering from gastroenteritis (GE): Biscaro *et al.* in Italy in 2018, Arowolo *et al.* in Nigeria in 2019, and Lekana *et al.* in Gabon in 2015 [13] [15] [16]. The frequency of RVA detection found differs from the 65.8% found by Giri Sidatha and his colleagues in India in 2020 during their study on the diversity of RVA genotypes circulating among children under 5 years of age hospitalized for GE [45]. This study demonstrated a significant frequency of RVA in the stools of children suffering from AFP, with an irregular distribution in some Regions of Cameroon compared to others. The Far North Region is the most conducive for the study of RVA, 19.7% (15/76). This observation can be explained by the fact that in the Far North Region, during the rainy seasons, frequent flooding leads to the pollution of drinking water with sewage. This observation, which explains the susceptibility of this Region of Cameroon compared to other Regions, has already been demonstrated by Ateudji and colleagues in 2019 to explain the repeated outbreaks of faecal-borne diseases such as cholera [46]. Specifically, regarding the Central Region of Cameroon, where several studies on the aetiology of GE cases have been conducted, an RVA detection rate of 10.5% was reported in this study. This result is lower than that of Boula *et al.*, who, when analysing the stools of children with GE admitted to the Yaoundé Mother and Child Centre between 2007 and 2012, found a detection rate of 41% [47]. The difference between the two studies is due not only to the sociodemo-

graphic and inclusion criteria of the participants (GE versus PFA), but also to the detection methodologies used: the ELISA technique for the previous study and PCR for this study.

This study highlights a significant correlation between RVA detection rates and variables such as age, sex, and year of detection, suggesting that these factors may have contributed to increased RVA positivity. The link between infant age and vulnerability to enteric virus infection affecting the nervous system has already been documented by other researchers around the world [5] [21] [22] [44] [45]. Notably, children between the ages of two and six were the most susceptible to developing symptomatic RVA infections. This can be explained by the fact that before the age of two, a large proportion of children still benefit from passive immunity conferred by their mothers through breastfeeding. When they are weaned, maternal antibodies gradually disappear, and active immunity gradually develops. From the age of two, children are more exposed to enteric infections. The main reason is that children start eating food and drinking water, which exposes them to food- and water-borne diseases. Our study revealed a higher incidence of RVA infection among male children compared to females, indicating greater susceptibility in boys. This observation has been reported in other contexts around the world, notably in a previous study demonstrating that male children have more structural malformations at the organogenesis stage than female children, for example, Hirschsprung's disease and tetralogy of Fallot [48]. This trend aligns with findings from other studies conducted globally, which have similarly reported a higher vulnerability to RVA infection among male pediatric populations [49] [50]. The monthly distribution showed a yearly circulation with peaks of detection during the rainy seasons. This observation on seasonality has been described by other authors [51] [52]. Heavy rainfall, therefore, appears to be a factor that promotes the transmission of RVA. However, another study conducted in neighbouring Nigeria in 2018 by Arowolo and his colleagues showed that enteric virus circulation was predominantly during the dry season [15]. These observations suggest that the seasonality of RVA infection varies depending on the study populations and epidemiological contexts.

In spite of expectations based on threshold cycle (TC) values, only 47.4% (36/76) of the samples with TC ≤ 36 were successfully amplified during genotyping. This amplification rate is notably lower than the 50% reported by Cunliffe *et al.* in 2000, suggesting potential differences in sample origin or assay performance. One plausible explanation is the presence of divergent viral strains refractory to the primer sets used, justifying the reduction of amplification efficiency. Furthermore, the use of conventional RT-PCR, which is known to be less sensitive than real-time PCR, may have contributed to these limitations in typing [53]. The discrepancy between the two studies may be primarily explained by differences in study design and patient inclusion criteria: while our investigation relied on analysis of children presenting with acute flaccid paralysis (AFP), the other study focused on patients with severe diarrheal symptoms. Another plausible explanation for the failure of

RT-PCR typing in certain samples is the presence of divergent viral strains that do not match the primer systems employed. Additionally, the lower sensitivity of conventional PCR compared to real-time PCR may have contributed to the amplification failure during the genotyping process. Although several RVA-positive samples could not be successfully typed, our findings nonetheless underscore the widespread circulation of Rotavirus A (RVA) in Cameroon. Phylogenetic analyses of the sequences generated from these amplicons revealed a high genetic diversity of RVA in Cameroon. The different genotypes obtained were either unique genotypes or genotypic combinations. In total, 77.7% (28/36) of the RVA detected by real-time PCR could be genotyped. The G1 genotype of RVAs was the most common, at 82.1% (23/28). The other genotypes obtained were: 3.6% (1/28) and 14.3% (4/28) for the G1P8 genotype combination alone. This high rate of G1 RVAs is higher than the rate of 44.9% found in the diarrheal stools of children under 5 years of age in another study conducted in Cameroon by Esona and colleagues in 2010 [54]. This high rate of RVA G1 is due to the fact that the vaccine currently used in Cameroon since 2014 to protect children against RVA infections is Rotarix, which provides incomplete protection against other rotavirus strains, including RVA G1, which triggers partial immunisation against these strains, allowing them to evade immunity. These variants therefore continue to circulate and infect individuals, even those who have been vaccinated. Under this selection pressure, less effectively neutralised rotavirus G1 strains gain a competitive advantage, which promotes their maintenance and spread within the population, thereby limiting the impact of vaccination on their prevalence. Rotarix is monovalent and does not target the RVA G1 strain but rather the G1P8 strain [22]. These genotypes have been described in the literature by several other authors [32] [47] [55] [56]. The G1P8 genotypic combination was characterised in this study at 14.3%. This low detection frequency is due to the high vaccination coverage of children against the RVA G1P8 strain, which is specifically targeted by the Rotarix vaccine used by Cameroon's Expanded Programme on Immunisation (EPI) [57] [58]. The genetic proximity of the sequences in this study to the MH933785.1 sequence of RVA G1P8 isolated in Cameroon in 2014 shows that this strain has been circulating in Cameroon for several years. However, they are distant from the other RVA genotypes that circulated in Cameroon in 2009 and 2014 (**Figure 4**). This study reported a very low average intragenotypic genetic diversity for rotaviruses (RVA) of 0.2 (SE: 0.001) for genotype G1 (**Table 3**), indicating the circulation of closely related viruses. These results are identical to those of Jelle Matthijnssens and colleagues, who reported a general threshold value of 0.2 (20% divergence) for a strain to belong to a given clade [59]. However, the genetic distances between the reference sequences of the G1 and those of the study gave values of 0.03 (SE: 0.006), 0.0179 (0.009). These calculated values are less than or equal to the threshold of 0.2. This supports the hypothesis that the RVA sequences studied do indeed belong to the genetic lineages described above. The average intra-genotype distances between the study sequences from the same Region yielded

non-zero distances for the Far North, Littoral, and Adamaoua (0.005, 0.009, 0.004, respectively), indicating that they were indeed different variants, although they define approximately the same strain that would have circulated in these Regions in a likely epidemic mode (**Table 3** and **Table 4**). However, zero distances were reported for the East, West, North, North-West, and Central regions (**Table 4**), indicating that only one variant of the strain present was detected there. This study thus describes for the first time a potential silent viral epidemic in RVA that occurred in Cameroon in 2018 without being documented by the rotavirus surveillance system. The Rotavirus G1 strain spread rapidly among the population over a relatively short period of time. This epidemic is confirmed by the average genetic distances obtained between strains from certain Regions compared to others. As a result, some of the variants of the Far North strain were found in the North, West, East, and North-West for RVA G1. The circulation of the same viral strain during an epidemic for a given period of time in one or more Regions would have been associated with a one-off, temporary epidemic. Comprehension of the mechanisms underlying the spread and persistence of RVA is vital for its effective control and eventual elimination in human populations. This study provides the first dataset from Cameroon on the spatio-temporal circulation of RVA. A positive correlation coefficient of 0.7 was observed between genetic divergence and the duration of virus circulation, supporting the application of Bayesian phylogenetic methods (**Figure 5**). The analysis revealed an average evolutionary rate of 9.13×10^{-4} substitutions per site per year. This value aligns well with previously reported estimates for the RVA genome, which ranged from 5×10^{-4} to 2.8×10^{-3} substitutions per site per year, as documented by Morozova *et al.* (2020) [42]. Phylogeographic analyses have traced the dissemination of RVA across various Regions of Cameroon. Findings suggest that the most probable ancestral strain originated in Mozambique around 2014 and was introduced into Cameroon in 2017 (**Figure 6**). The introduction of this imported virus, based on the data generated in this study, constitutes a single introduction event and cannot be considered part of a broader, ongoing regional circulation pattern, given the scarcity of research on this subject. This study is the first to describe the circulation of this virus in Central Africa and Cameroon in particular. Previous research by Motayo *et al.* (2019) identified Mozambique as a central epicentre for RVA epidemics, which rapidly extended to both neighboring and distant nations, likely facilitated by international travel and economic exchanges within the African continent [60]. Within Cameroon, the Littoral Region emerged as a key hub of RVA transmission, serving as the principal source of viral spread to other urban centers from 2017 until its detection in 2018 during this study. Considering the significant geographic separation between Cameroon and Mozambique, as well as among the Cameroonian Regions, these observations suggest that RVA transmission is primarily sustained through the mobility of infected individuals or asymptomatic carriers moving across borders and between regions.

Supporting evidence from earlier studies indicates that RVA transmission chains

among human populations are relatively short, yet sufficient to enable long-distance spread [1] [45] [60]. This accelerated transmission may be attributed to the virus's environmental resilience, its ability to remain viable on surfaces under varying conditions, and its low infectious dose threshold. Notably, up to 90% of the infectious inoculum remains present after 60 days [43]. Additional multidisciplinary investigations underscore the role of interregional human movement in facilitating the spread of RVA over large distances [61].

5. Study Limit

This study had several limitations related to the non-use of next-generation sequencing, which did not allow for the expansion of the genetic diversity of rotaviruses detected, due to the sensitivity of the technique and the alternative methodology used in this study. Viruses with low viral loads or present in reduced proportions in the samples could not be fully characterised. The objective of fully sequencing the genomes of the identified genotypes was compromised by the low viral load of some samples, resulting in incomplete and partial coverage of the genomes. This limitation reduced the understanding of genetic recombination mechanisms and the detection of recombinant variants. Furthermore, the lack of systematic clinical assessment of rotavirus-related morbidity and mortality in children with severe neurological infections (meningitis, encephalitis) limited the assessment of the real impact of these pathogens on paediatric health. Despite these constraints, the study provides a robust, albeit incomplete, estimate of the diversity and potential role of rotaviruses in human pathology in Cameroon, while opening up clear prospects for more targeted future research.

6. Conclusion

This study is the first investigation in Cameroon to detect RVA in the stool samples of children presenting with acute flaccid paralysis (AFP). The findings suggest that a diverse range of RVA genotypes circulates year-round among this pediatric population. Notably, this study documented the emergence of the RVA G1 genotype during a previously unrecognized outbreak in 2018. An event overlooked by national public health surveillance systems. This outbreak is likely linked to the administration of a vaccine under the Expanded Program on Immunization that failed to target the full spectrum of RVA strains circulating in the Cameroonian community. The evidence presented in this study offers a compelling rationale for the Ministry of Public Health to consider supplementing the current vaccine with broader-spectrum formulations such as RotaTeq. It further underscores the urgent need for national health authorities and the World Health Organization to address the growing threat posed by RVA G1, particularly in Cameroon's predominantly children under 15 years of age. To strengthen viral control measures, the study advocates for integrating RVA surveillance into the existing AFP monitoring framework.

Ethical Approval

Informed consent was not applicable for this retrospective study. Ethical derogation was granted by the Centre Regional Ethics Committee of Research for Human Health (CRERSHC) in Cameroon under approval number CE No. 2374/CRERSHC/2021, permitting the retrospective use of specimens and associated data collected as part of the poliomyelitis surveillance program in Cameroon, which is supervised by WHO surveillance standards WHO/CDS/CSR/ISR/99.2.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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