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Original Article

Occurrence of poliovirus and non-polio enterovirus among children with acute flaccid paralysis in Cameroon from 2015 to 2020

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Abstract

Introduction: Poliovirus (PV) and non-polio enteroviruses (NPEV) belong to the Picornaviridae family. They are found worldwide and are responsible for a wide range of diseases such as acute flaccid paralysis (AFP). This study aimed to evaluate the detection rate of PV and NPEV in stool samples from children under fifteen years of age presenting with AFP in Cameroon and their distribution over time.

Methodology: Stool samples were collected as part of poliovirus surveillance throughout Cameroon from 2015 to 2020. Virus isolation was performed using RD and L20B cells maintained in culture. Molecular methods such as intratypic differentiation were used to identify PVs serotypes and analysis of the VP1 genome was performed.

Results: A total of 12,354 stool samples were analyzed. The EV detection rate by virus isolation was 11.42% (1411/12354). This rate varied from year to year with a mean distribution of 11.41 with a 95% confidence interval [11.37; 11.44]. Of the viruses detected, suspected poliovirus accounted for 31.3% (442/1411) and NPEV 68.67% (969/1411). No wild poliovirus (WPV) was isolated. Sabin types 1 and 3 were continuously isolated. Surprisingly, from February 2020, vaccine-derived PV type 2 (VDPV2) was detected in 19% of cases, indicating its resurgence.

Conclusions: This study strongly supports the successful elimination of WPV in Cameroon and the resurgence of VDPV2. However, as long as VDPV outbreaks continue to be detected in Africa, it remains essential to monitor how they spread.

Key words: Poliovirus; enteroviruses; acute flaccid paralysis; Cameroon.

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Introduction

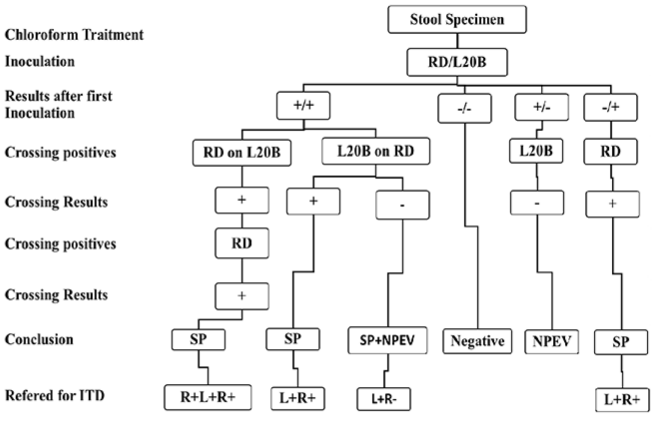
Acute flaccid paralysis (AFP) is a clinical syndrome characterized by rapid onset of weakness in one or more limbs and weakness of the respiratory and digestive muscles [1]. AFP could be of infectious and non-infectious origin [2]. Among the infectious causes, the most likely is enterovirus (EV) [3]. EVs are small, non-enveloped viruses that infect humans and animals [4]. In humans, EVs are ubiquitous and cause significant morbidity and mortality in both adults and children [5]. Each year, approximately one billion infections are reported worldwide [6]. EVs are transmitted through the faecal-oral route and also through direct or indirect contact with contaminated objects or aerosols [7].

There are over 116 EV serotypes, which can be grouped into poliovirus (PV) and non-polio EVs (NPEV) [8]. PV is the prototype virus of the EV genus and there are three types of PV namely PV-1, PV-2 and PV-3 [9]. Each of these types can be either wild-type PV (WPV) or True Sabin-like (SL). PV is the causative agent of poliomyelitis and due to the severity and devastating nature of this disease, the World Health Organization (WHO) initiated the Global Polio Eradication Initiative (GPEI) in 1988 [10]. The GPEI strategy included mass immunization of at-risk populations, surveillance of AFP cases, and environmental surveillance of PVs. The GPEI program has led to a remarkable reduction in poliomyelitis cases. From a peak of 350,000 cases in 1988, to only 11 cases in Afghanistan and Pakistan in the second quarter of 2022 [11]. On May 15, 2022, a case of WPV1 was detected in Mozambique [12]. This case was similar to one reported in Malawi in February 2022 and was genetically related to a strain that would have circulated

in Pakistan in 2019. The program owes its success to mass immunization with the oral attenuated polio vaccine (OPV). Although OPV confers greater immunity, once in the body, it can undergo genetic modification and transform into a virus, derived from the one used in vaccination (VDPV), causing diseases such as WPV. VDPVs can be classified as circulating VDPV (cVDPV), ambiguous VDPV (aVDPV) and VDPV detected in immunocompromised individuals (iVDPV) [13]. VDPVs have been the cause of several AFP outbreaks [14]. In 2022, the African region recorded: cVDPV1 epidemics in countries such as Malawi, Madagascar, the Democratic Republic of Congo, and Mozambique; cVDPV2 epidemics were reported in Algeria, Benin, Botswana, Burkina Faso, Cameroon, Central African Republic, Chad and Cote d'Ivoire [15].

In Cameroon, the last cases of WPV1, WPV3, and VDPV cases were reported in 2014, 1999, and 2013, respectively [16,17]. There have been no reports of WPV2 so far. In April 2016, Cameroon replaced the use of the trivalent OPV (SL types 1, 2, and 3) with the bivalent OPV (SL types 1 and 3) and introduced a dose of inactive PV vaccine type 2 in routine vaccination [18]. Despite no new reports of wild-type PVs in the country, the burden of AFP remains high. To investigate alternative causes of AFP, we sought to evaluate the PVs and NPEV detection rate and the temporal distribution of different types of PVs recovered from stool samples of children under fifteen years of age presenting with AFP in Cameroon over the last six years (2015 – 2020).

Figure 1. Viral isolation block diagram.



Poliovirus detection algorithm; RD: Human rhabdomyosarcoma cell; L20B: Mouse kidney cell expressing human PV receptor; (+): CPE positive; (-): CPE negative; SP: Poliovirus suspected; Isolate type: R + L + R +; L + R +; L + R -; NPEV: Non-polio enterovirus.

Methodology

Study population

Our study is retrospective and cross-sectional conducted from January 01, 2015, to December 31, 2020. Children aged < 15 years of age presenting with acute flaccid paralysis (AFP) at any health facility in Cameroon were included in the study. A questionnaire was used to record the following patient's information: date of paralysis, clinical symptoms, symmetry location, and onset of paralysis. For each patient, two stool samples were collected 24-48 hours apart within 14 days of paralysis onset. Samples were transported to Centre Pasteur du Cameroun (CPC), where they were carefully kept in a cold chain (4–8 °C) for virological analysis. Prior to sample collection, verbal assent was obtained from each of the participant's parent or guardian.

Cell lines and virus isolation

At the CPC, stool samples were processed for virus isolation according to WHO protocols. Briefly, stool samples were treated with chloroform to kill bacteria, fungi and to dissociate viral aggregates and then centrifuged at 1500 g for 20 minutes. The resulting supernatants were inoculated into four cell culture tubes, two containing RD cells (human rhabdomyosarcoma) and the other two containing L20B cells (mouse kidney cells expressing the human PV receptor). The cells were maintained in minimal essential medium (MEM) supplemented with 2% fetal calf serum, 1% Penicillin Streptomycin Neomycin antibiotic, 2.5% bicarbonate, 1% hepes, and 1 mM L-glutamine. Culture tubes were incubated at 36 °C in a 5° stationary-inclined position. Tubes were examined daily by microscopy for cytopathogenic effects (CPE). L20B-positive cells were further passaged into RD cells. If CPE occurred, the specimen was reported as positive (L + R +) and referred for intratypic differentiation (ITD). RD-positive cells were passed in L20B and, if positive, repassaged in RD. If CPE appeared in this RD passage, the specimen was reported as positive (R + L + R +) and further referred for ITD. After tube reading, all samples with CPE on the L20B line, regardless of the arm, were reported as suspect poliovirus (SP) and stored frozen (-20°C) for molecular characterization. Samples with observed CPE only on the RD cell line and not on L20B cells were considered and reported as NPEV. All inoculated samples without CPE after 10 days of reading were reported as negative for EV isolation (Figure 1).

Molecular characterization of PVs

To verify whether the isolates with CPE on L20B were PV or not, and to determine the nature (vaccine or wild-type) of all PV isolated, the isolates were analyzed by ITD, which is a molecular detection method using two sets of 8 reverse polymerase chain reaction (rRT-PCR) with specific primers and probes. The first set of 6 rRT-PCR served to identify and differentiate the wild-type poliovirus. The second set of 2 rRT-PCR was used for VDPV screening according to the WHO protocol [19]. The list of primers, probes, and the running program used in this study was described by Nancy and collaborators in 2018 [20]. Briefly, 1 µL of control RNA (or) 1 µL of supernatant for each sample is mixed with 19 µL of reaction solution (10 µL of Tough Mix, 8 µL of water and 1 µL of PV-specific primers and probe) into the appropriate reaction well. The amplification program is summarized as follows: 50°C for 30 minutes (RT reaction), 95°C for 1 minute (inactivation of RT), and two-stage PCR cycles (95°C for 15 seconds, 50°C for 45 seconds (collection of endpoint fluorescence data), then 25% ramp speed and 72°C for 05 seconds while running 40 cycles. The curves for each sample were compared with the positive and negative controls for reference. All PV 1 and 3 obtained from the first set of rRT-PCR were tested for VDPV using the second set of primers and probes.

All isolates with indeterminate results (Pan EV and Pan PV positives and negative for other assays) and non-reactive with the second set of rRT-PCR, were sent for VP1 analysis [20].

Since the withdrawal of type 2 OPV from routine immunization in 2015, all detected PV2 have been systematically sent for full-length VP1 capsid coding gene analysis [21].

Following WHO recommendations, phylogenetic analyses were carried out by the Center for Disease Control, and the results were sent to the requesting laboratory in the form of an Excel file. The file contained information on genetic proximity, percentage similarity to Sabin 2 used in vaccination, and origin from 2015 to 2020.

Data analysis

Statistical analyses were performed using Epi Info version 3.5.4 and Excel software.

Ethical consideration

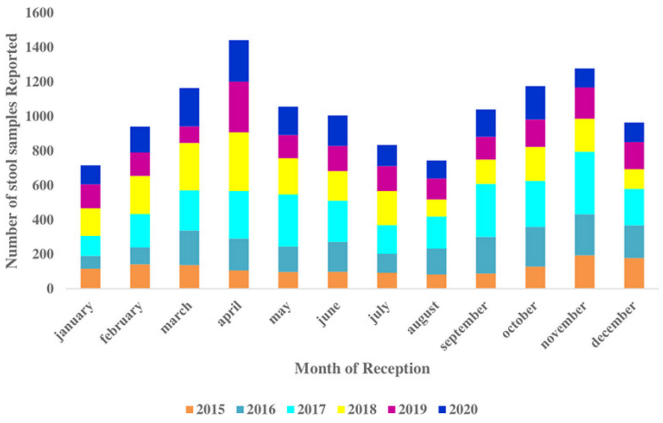
This study received approval from the Cameroon Centre Regional Ethics Committee for Human Health Research (CRERSH/C) Number: CE N° 2374/CRERSHC/2021 for utilization of data collected from the Polio virus surveillance for research purposes.

Results

A total of 12,354 stool samples were collected between January 2015 and December 2020. Approximately 33% of the samples were from the Far North region of the country. Sampling was regular throughout the study period, with the highest number of samples collected during the rainy season: March to May and September to November (Figure 2).

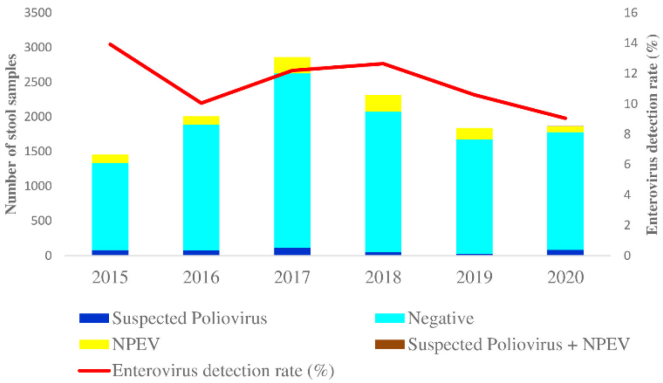
After inoculation of the stool supernatant, 1411 samples showed CPE on cell lines, only 31.32% (442/1411) of the samples showed CPE on both RD and L20B cell lines. Of these, 99.8% (441/442) were suspected poliovirus (SP) and 0.2% (1/442) were a

Figure 2. Number of Stools Samples Reported by month from 2015-2020.



Breakdown of saddle samples received by month: on the ordinate the samples received and on the abscissa the months in which they were received.

Figure 3. Enterovirus isolation rate from stool by year.



Isolation results per year: ordinate: number of samples received, abscissa: years of viral isolation secondary ordinate: enterovirus detection curve per year.

combination of SP and NPEV. This co-infection was detected for the first time in 2020 in the Far North region of the country. Overall, 68.68% (969/1411) of samples showed CPE only on the RD cell line (NPEV) (Figure 3).

EVs in their globality were detected by isolation in 11.42% (1411/12345) of samples, with an annual average detection at a 5% risk threshold of 11.41 [11.37; 11.44]. The EV detection rate varied from year to year with a maximum of detection (13.92%) in 2015 and a minimum (9.05%) in 2020 (Figure 4). The distribution of EVs detection varied from one region to another. The Center region of Cameroon has the lowest detection rate (6.44%) and the Far North of the country, has the maximum level (14.11%) followed by the Littoral region (12.57%) (Figure 3).

By observing the variation in the frequency of EV detection in relation to the years in which the samples were received, we observed that the rate of EV detection progressively decreased with the years. Our hypothesis was verified by examining the association between the two types of data using the Chi-square test. A statistically significant P-value of 0.0178 was obtained.

A total of 999 NPEVs isolates (8.08%) were obtained from 12354 stool samples collected. Of the NPEVs obtained, 97% (969/999) gave CPE only on the RD cell line and 3% (30/999) gave CPE on both cell lines (RD and L20B). The detection of NPEVs by isolation was highest in the year 2018 (10.36%), whereas, the year 2020 had the lowest detection rate (4.52%). In 2017, the year with the highest number of samples, the detection rate of NPEVs was not the highest (Figure 5).

After molecular characterization, a total of 577 virus types were detected. These viruses were divided into five main groups: 6 NEV (1.03%), 39 NPEV (6.75%), 519 Sabin viruses including 402 SL1 and SL3 (69.7%) and 114 SL2 (19.7%). VDPV2 (2.25%) was further divided into 02 aVDPV2 (0.34%) and 11 cVDPV2 (1.9%) (Figure 6). The SL2 viruses detected in our study were found in all regions of the country. VP1 analysis of their genome showed that they differed by 0 and 1 nucleotides from the reference SL2 strain used for vaccination (99-100% similarity). All SL2 isolates were therefore vaccine derived.

Figure 5. Frequency of detection of NPEV among Stool samples collected from 2015 to 2020.

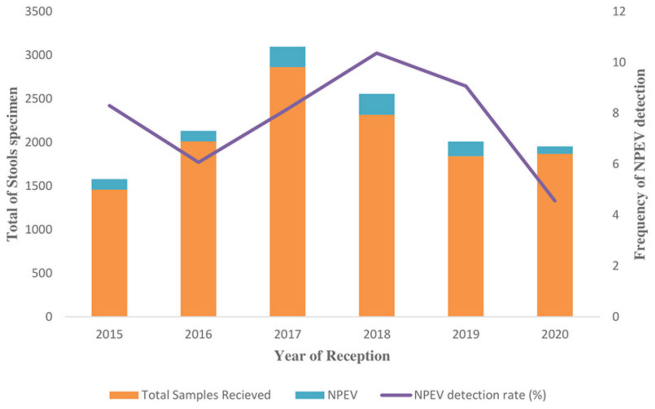


Figure 4. Number of samples analyzed and isolation results by regions by years between 2015 and 2020.

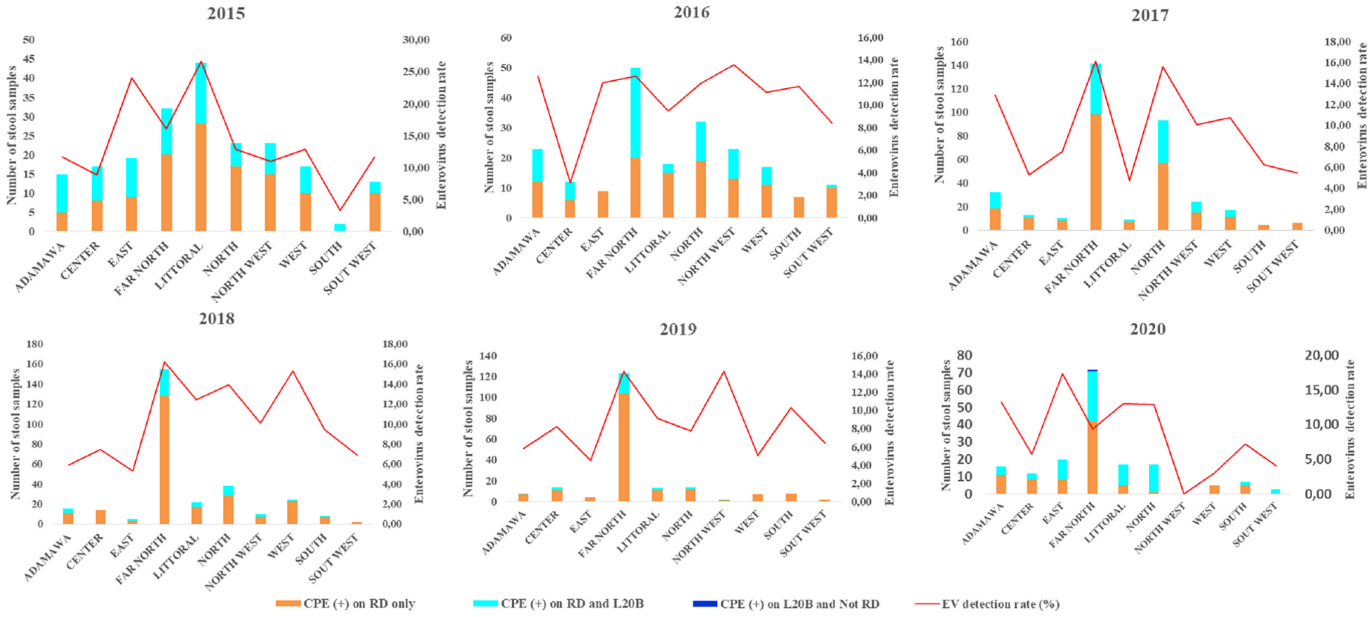
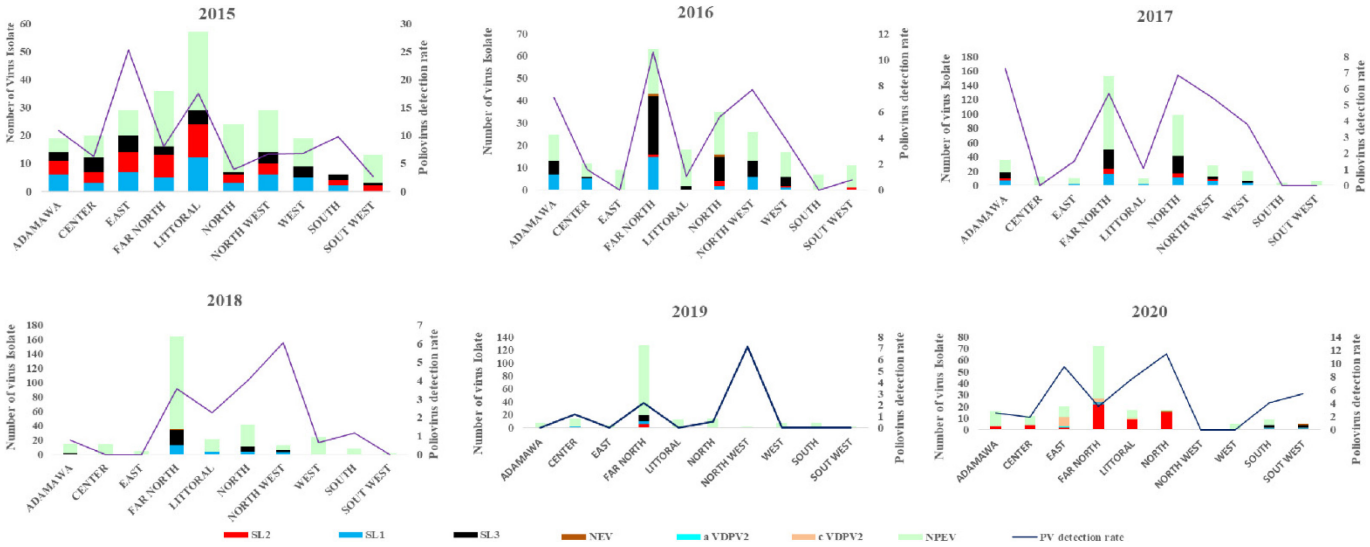


Figure 6. Number of samples analyzed and virus types detected by ITD in the resulting isolates from Cameroon regions between January 2015 to December 2020.



Breakdown of virus isolates detected in ITD by year: green: NPEV, black: SL3, red: SL2, dark blue: SL1, purple: NEV, orange: cVDPV2, light blue: aVDPV2 and green: PV detection rate curve.

The detection of isolated VDVP2 strains was limited to three regions of Cameroon (East, Littoral, and Far North) (Table 1). The VDPV2 isolates from the Eastern region were derived from the reference SL2 used for vaccination. Phylogenetic analysis of their conserved region showed that they were closely related to strains circulating in 2020 both in humans in this region and in the wastewater environment of another region of the country (ENV-CAE-SUD-EBO-DGN-20-010) and the neighboring Central African Republic (ENV-CAF-RS2-BOB-PAY-20-003) . VDPV2s from the coastal region of Cameroon differed from the reference SL2 by only 10 nucleotides. For the Far North region of the country, the VDPV2s found were 83 to 85% similar to the reference SL2. They were closely related to the strains that circulated in Chad between 2019 and 2020, both in the wastewater environment (ENV-CHA-NDJ-CEN-CPR-19-023) and in humans (CHA-TAN-DON-20-396) respectively (Table 1).

Unlike the NPEVs which were detected throughout our study period regardless of their origin and month of collection, PVs were only detected in certain months of our study period. The months of January, May, and November in 2015; February, and June to August in 2016; January, July, August, and December in 2017; January, May, June, August, September, and November in 2018; January to March, August, and September to November in 2019; then May, July, August, and December in 2020. This diverse distribution perfectly illustrates the irregular annual circulation of PVs (Figure 7). Although a seasonality model could not be defined from our study period, several differences in

Table 1. Origin of detection of VDPVs detected in Cameroon from 2015 to 2020.

Region	VDPV2 Sequencing Results	Stool Collection Date	District of Origin	Closest match	Percentage match with Sabin 2	Date of last OPV	Total OPV doses
EAST	cVDPV	06/02/2020	Betare Oya	ENV-CAF-RS2-BOB-PAY-20-003	80		
	cVDPV	17/02/2020	Garoua-Mboulaï	CAE-EST-BET-20-036	82	18/11/2018	
	cVDPV	29/03/2020	Betare Oya	CAE-EST-GAB-20-052	75		
	cVDPV	29/03/2020	Betare Oya	CAE-EST-GAB-20-052	75		
	cVDPV	29/03/2020	Betare Oya	ENV-CAF-RS2-BOB-PAY-20-003	83	28/05/2009	3
	cVDPV	28/03/2020	Betare Oya	CAE-EST-BET-20-036	79	14/04/2017	
	aVDPV	16/04/2020	Kette	ENV-CAF-RS2-BOB-PAY-20-003	85	02/03/2019	1
	aVDPV	17/04/2020	Kette	ENV-CAF-RS2-BOB-PAY-20-003	85	02/03/2019	1
	cVDPV	31/08/2020	Batouri	ENV-CAE-SUD-EBO-DGN-20-010	81		3
LITTORAL	cVDPV	11/03/2020	Bangue	Sabin 2 Reference strain	90		
FAR NORTH	cVDPV	18/09/2020	Maroua 2	ENV-CHA-NDJ-CEN-CPR-19-023	85		10
	cVDPV	19/09/2020	Maroua 2	ENV-CHA-NDJ-CEN-CPR-19-023	85		10
	cVDPV	20/09/2020	Vele	CHA-TAN-DON-20-396	83	01/10/2018	6

The percentage similarity of VDPV2 detected with SL2 used for vaccination is given in the table. cVDPV is circulating VDPV and aVDPV is ambiguous VDVP.

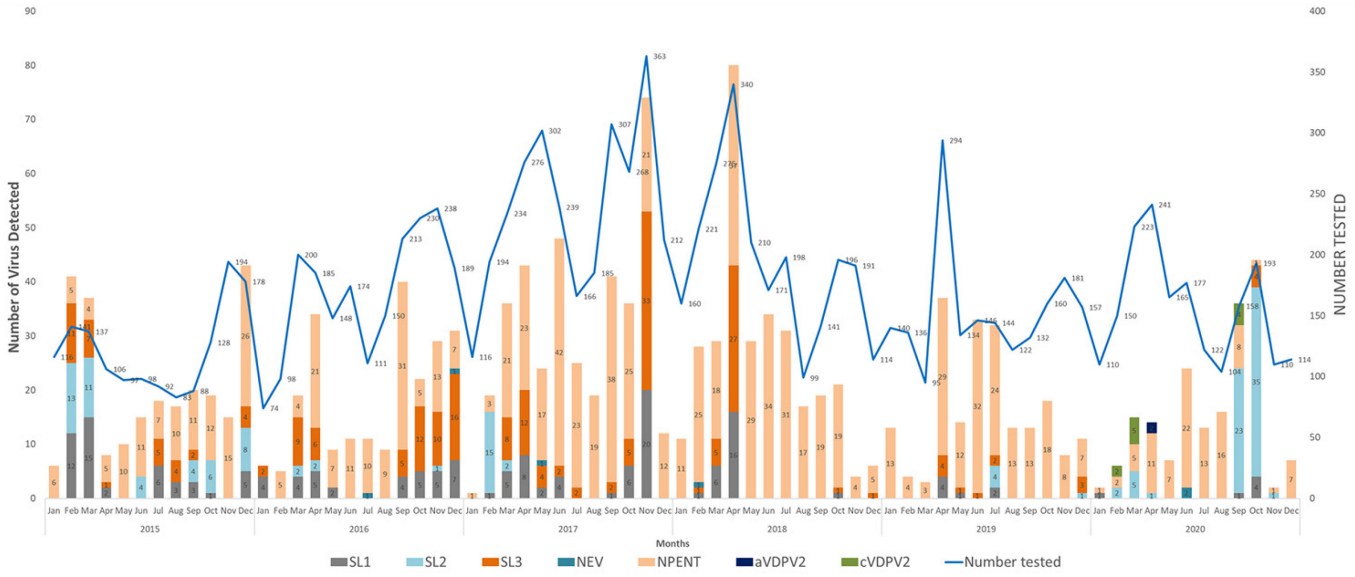
virus distribution were detected in all regions of the country. All SL were isolated throughout the study period, but their temporal distribution appeared to be more dispersed in the North West region (5.92%) compared to 1.64% in the South West region of the country (Figure 7). Several regions of the country did not isolate PVs during our study: the East region in 2016, the central region in 2017, the two regions mentioned above in 2018, the East region in 2019 and the West and North West regions in 2020 (Figure 6). More specifically, the SL2 showed the same temporality in all regions of the country (Figure 7). Their distribution was observed at the same time as the detection of other PVs.

Of the 97 isolates submitted for the VP1 sequence analysis, 86% (84/97) showed no nucleoside differences with the SL2 used in the vaccination, confirming their Sabine status (Table 1). Thirteen (13) isolates from the year 2020 were thus confirmed as VDPV. Of these, 11.3% (11/97) were cVDPV2 and circulated during February, March, and September; while 2.1% (2/97) isolates were aVDPV2 and circulated during April (Figure 7). These results provide substantial support that WPV transmission has been successfully interrupted in Cameroon since 2014, but calls on the national and international community against VDPV2 resurgence.

Discussion

This study is the first to investigate EV occurrence in Cameroon from 2015 to 2020. We reported an EV detection rate of 11.4%, which suggests that a large proportion of samples (88.6%) was negative for EV isolation. This observation contradicts that of Lieke Brouwer and colleagues, who reported an EV detection rate of 89.9% in stool samples from Malawian children. This difference can be explained by the fact that, the latter study used molecular methods with the primary intention of screening EVs, whereas we used viral isolation to detect EVs [22]. On the other hand, our study reported a strong negative correlation between the frequency of detection of EVs and the years of stool collection, suggesting a decrease or even a definitive absence of circulation of these PVs in the coming years. This is the aspiration of the GPEI program, which aims to eliminate PVs in Africa by 2026 [23]. This study shows that the detection of EV in stool samples does not depend on any socio-demographic factor. The same observation and the absence of an association between age and EV detection were obtained by Mohsan Saeed and collaborators while investigating the epidemiology and clinical findings associated with enteroviral AFP in Pakistan from January to December 2003. NPEV was detected at a rate of 8.08% in our study. This result is higher than the 7.2% reported by Hugo Kavunga Membo and collaborators while studying AFP

Figure 7. Temporal distribution of the isolation of non-polio enteroviruses, vaccine and derived polioviruses in Cameroon from January 2015 to December 2020.



Temporal distribution of isolation of vaccine-derived polioviruses and non-polio enteroviruses in Cameroon, from January 2015 to December 2020. The number of individual viruses detected (SL1: Sabin type 1; SL2: Sabin type 2; SL3: Sabin type 3; NPEV: non-polio enterovirus; NEV: non-enterovirus; cVDPV2: circulating vaccine-derived poliovirus2; aVDPV2: ambiguous vaccine-derived poliovirus2) isolated and the number tested are shown by month.

surveillance indicators in the Democratic Republic of Congo from 2008 to 2014. Due to the continuous isolation of NPEV from AFP stool samples throughout the study period, our study confirms a typical circulation of these viruses in Cameroon as previously described [24]. The statistical analysis of the association between AFP cases and NPEV isolation revealed the absence of an association between these two events. SL (1,2,3) detected during our study is the consequence of using these viruses in the framework of the extended vaccination program in Cameroon. Our study reports the absence of WPV circulation during these last 6 years in Cameroon, thus confirming the eradication of WPV in our country and in Africa [25]. No WPV type 2 has been reported in our study since the official announcement of its elimination in 2015, suggesting that the eradication of this virus in our country is effective, as declared by the WHO global initiative for the eradication of PV in Africa. In Cameroon, the last case of VDPV2 was detected in 2013, circulating in the Far North region of the country [16]. Until January 2020, there was no detection of these viruses, suggesting the absence of their circulation. From February to end of 2020, VDPV2 re-emerged in Cameroon, thus testifying to a mutation of SL2 and its instability in the environment formerly used for vaccination [26]. In contrast to Cameroon, between 2015 and 2019, VDPV2 was reported in many countries, including Afghanistan, Nigeria, and Pakistan, leading to a reduction in population immunity to the SL2 used for vaccination [27]. It remains very important to maintain high quality AFP and environmental surveillance to ensure rapid detection and control of VDPV re-emergence and limit the consequence of their pathogenicity in humans after several efforts made by the Global Polio Eradication Initiative and governments to eradicate WPV in Africa.

Conclusions

The present study suggests that despite the AFP surveillance system set up to detect EVs, only a small number of viruses (11.42%) were detected. The data analyzed during our study confirm that the circulation of NPEV is regular throughout the year, whereas PVs are not. Our results indicate that there is no association between the detection of EVs and other factors such as year of collection or AFP cases. Our study confirmed the absence of circulation of WPVs, but showed the re-emergence of VDPV2, the last circulation of which dates to 2013. The data obtained on these vaccine-derived viruses will probably allow the formulation of more effective strategies, such as modification of the vaccines currently used in routine immunization, for their control and eradication in Africa. Studies on NEV isolates and a large proportion of samples negative for EVs by viral isolation could provide a better understanding of the epidemiology of these viruses other than EVs associated with AFP, and generate data on their circulation and their involvement in human pathology.

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