Early detection of vaccine-derived poliovirus outbreaks using nested PCR and nanopore sequencing in the Democratic Republic of Congo, 2021-2022

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Abstract

Delayed detection of poliovirus outbreaks is a major threat to polio eradication. Direct molecular Detection and Nanopore Sequencing (DDNS) of stool samples shows promise as a faster method to detect and confirm polio cases compared with cell culture but has not been assessed prospectively during routine surveillance. We report on the implementation of prospective testing of all stool samples received from suspected polio cases and their contacts in the Democratic Republic of Congo between 10th August 2021 to 4th February 2022. DDNS detected polioviruses in 62/2339 (2.7%) of samples whilst the standard algorithm of cell culture, qPCR and Sanger sequencing detected 51/2339 (2.2%). The sensitivity and specificity of DDNS was not significantly different from cell culture. DDNS provided the VP1 sequence required for case confirmation on average 7 days after sample receipt compared with 30 days for the standard algorithm, allowing detection of three new cVPDV2 outbreaks a mean of 23 days earlier (range 6-30 days) and was estimated to cost less per sample tested. The mean sequence similarity between sequences obtained by the two methods was 99.98%. Continued implementation of DDNS in DRC and expansion to other countries will allow further evaluation of this method and inform its potential recommendation by the Global Polio Laboratory Network.

Introduction

Despite the significant progress made by the Global Polio Eradication Initiative (GPEI) since 1988, poliomyelitis, a debilitating infectious disease due to poliovirus, remains a major public health problem in countries where there is low vaccination coverage. Poliovirus outbreaks are responded to by mass vaccination campaigns with oral poliovirus vaccine (OPV) which aim to stop transmission before wider spread. However, delayed shipping of stool samples, time-consuming and complex laboratory methods based on virus isolation in cell-culture and limited availability of sequencing capacity can delay outbreak response and thus reduce the impact of mass vaccination campaigns [1–3].

In August 2020, the African Region was declared to have interrupted the transmission of wild poliovirus. Vaccination with OPV has, however, led to outbreaks of circulating vaccine-derived poliovirus (cVDPV) that can occur following reversion of attenuating mutations in the live-vaccine strain, which is shed following vaccination and can spread in under-immunised populations [4,5]. cVDPV2 epidemics in young children plague Africa and other regions in this post-wild poliovirus era. In 2020, 959 cases of paralysis caused by serotype 2 cVDPV (cVDPV2) were reported globally in 27 countries, including 21 countries in Africa[6]; in 2021, 692 cases caused by cVDPV2 and 20 cases by serotype 1 cVDPV were reported globally, the majority in Africa including Nigeria and the Democratic Republic of Congo (DRC)[7].

In DRC, ten years after the last case of Wild Polio Virus (WPV), there has been a situation of almost permanent circulation of cVDPV2 as a result of new emergences of cVDPV2 following the use of serotype 2 (Sabin) OPV in response to existing outbreaks. Poliovirus surveillance is based on the collection of stool samples from children with acute flaccid paralysis (AFP) and their contacts, and on environmental (sewage) sampling, and relies on timely and high-quality sample collection and laboratory testing. In

DRC, the proportion of AFP cases with adequate stool sample collection (where 2 stools are collected 48 hours apart, within two weeks of onset of paralysis and arriving by cold chain with proper documentation) was 77% in 2018[7]. Gaps in immunisation coverage, particularly due to low routine immunisation by IPV with an estimated 65% of infants were fully vaccinated in 2021[8,9], have put the DRC at high risk of additional cVDPV outbreaks. Logistical challenges in sample shipment to the laboratory, in laboratory testing of the samples and in international shipment (to South Africa) for sequencing cause delayed detection of new poliovirus outbreaks, with case numbers stemming from an outbreak increasing by approximately 12% per additional week[10]. The World Health Organisation (WHO) identify delays in detection as one of the major challenges facing the eradication programme[11].

The DRC covers a large area of 2,345,000 Km² where health indicators remain of concern and a single WHO accredited laboratory at the Institut National de Recherche Biomédicale (INRB) in Kinshasa is responsible for country-wide biological diagnosis of poliovirus. The laboratory uses a sensitive and highly standardised WHO detection protocol that involves cell culture followed by intratypic differentiation (ITD) qPCR. Sequencing of the key poliovirus VP1 capsid region is performed at a separate specialised laboratory in the Republic of South Africa. A VP1 sequence is required to confirm each poliovirus detection or case and to distinguish cVDPV from vaccine strains.

The GPEI is at a crossroads and is considering new approaches to achieve polio eradication in the last two wild poliovirus-endemic countries, Afghanistan and Pakistan, and to combat outbreaks of cVDPV in four of WHO's six geographical regions [3]. The World Health Organisation Polio Eradication Strategy 2022–2026 [3] commits the programme to improvements in detection and response. Two such aims are the implementation of direct detection of poliovirus in stool samples, which would remove the need for the cell-culture based detection algorithm according to the world-wide poliovirus containment aims [12], and the shifting of poliovirus testing and sequence analysis to country level.

These aims could be achieved through the implementation of the direct detection by nanopore sequencing (DDNS) method [13]. This method combines faster, direct detection from stool samples with on-site sequencing, avoiding time-consuming, international transport of samples and facilitating rapid detection of the chain of transmission and quick response to outbreaks [10]. It can be implemented in most laboratories familiar with PCR, including INRB where both Illumina and Nanopore sequencing have been used for Ebolavirus, measles, monkeypox and SARS-CoV-2 [14,15].

Here we present a prospective study evaluating the use of rapid, direct sequencing via the DDNS protocol alongside routine cell-culture methods for poliovirus surveillance in DRC. We report the sensitivity and specificity of DDNS compared with cell culture, sequencing accuracy, time-taken in the lab and associated cost data.

Results

DDNS results compared with the standard algorithm

Stool samples were tested in parallel via DDNS and the standard culture-based detection algorithm. 2,339 prospective stool samples were processed over 26 nanopore sequencing runs across the 141-day period that sequencing was performed, averaging one sequencing run every 5.4 days. DDNS testing found 62 samples (2.7 % of total samples) positive for poliovirus; 36 for serotype 2 VDPV (1.58 %), 5 for Sabin serotype 1 (0.30 %), 19 for Sabin serotype 3 (0.90 %) and 2 for both serotypes 1 and 3 Sabin poliovirus (0.09 %) (Table 1). The standard cell culture algorithm identified polioviruses in 51 samples; 31 samples testing positive for serotype 2 VDPV (1.33 %), 4 for Sabin serotype 1 (0.17 %) and 16 for Sabin serotype 3 (0.68 %).

Table 1 – Poliovirus detection by DDNS and the standard cell-culture, ITD and Sanger sequencing algorithm

		DDNS				
		Sabin 1	**VDPV2**	**Sabin 3**	**Sabin 1 + Sabin 3**	**Negative**
Cell-culture, ITD and Sanger sequencing	**Sabin 1**	**3**	0	0	0	1
	VDPV2	0	**27**	0	0	4
	Sabin 3	0	0	**15**	0	1
	Negative	2	9	4	2	**2271**

The sensitivity and specificity of detection for each poliovirus type was determined for DDNS versus the cell-culture algorithm and vice versa (Table 2). Additional cVDPV2 detected by either method were not identified as contamination as sequences differed from those of other samples (as shown in Supplemental Figure 1). The sensitivity and specificity of the two methods did not differ significantly (Fisher's exact test).

Table 2 – Sensitivity and specificity by sample for detection of Sabin 1 and Sabin 3 polioviruses and VDPV2 by the standard cell-culture algorithm versus DDNS and vice versa. P-values were generated using Fisher's exact test.

		Culture vs DDNS (95% CI, n/N)	DDNS vs culture (95% CI, n/N)	Test for difference between methods, P-value
Sabin 1	Sensitivity	43 (10-82, 3/7)	75 (19-99, 3/4)	0.55
	Specificity	100 (100-100, 2331/2332)	100 (100-100, 2331/2335)	0.37
VDPV2	Sensitivity	75 (58-88, 27/36)	87 (70-96, 27/31)	0.24
	Specificity	100 (100-100, 2299/2303)	100 (99-100, 2299/2308)	0.27
Sabin 3	Sensitivity	71 (48-89, 15/21)	94 (70-100, 15/16)	0.11
	Specificity	100 (100-100, 2317/2318)	100 (99-100, 2317/2323)	0.12

Two stool samples were available for 1,118 AFP cases, with 37 cases positive for poliovirus by either method. 18 cases had full concordance between both methods and both samples testing positive (see Table 3). There were no cases where both samples tested positive by cell-culture and yielded no positive DDNS result, while in 9 cases with positive DDNS results no poliovirus was detected by the cell-culture algorithm.

Table 3 – Concordance of the cell-culture algorithm and DDNS for the testing of paired stool samples

		DDNS		
		++	**+-**	**--**
Culture	**++**	**18**	0	0
	+-	3	**4**	3
	--	4	5	**1081**

A single sample or pair of samples were available for 1,159 AFP cases. The sensitivity and specificity of detection were calculated for each AFP case (see Table 4), and for only AFP cases where two stools were available (n =1,118 cases, see Supplemental Table 1).

Table 4 – Sensitivity and specificity by AFP case for detection of Sabin 1 and Sabin 3 polioviruses and VDPV2 by the standard cell-culture algorithm versus DDNS and vice versa.

		Culture vs DDNS (95% CI, n/N)	DDNS vs culture (95% CI, n/N)	Test for difference between methods, P-value
Sabin 1	Sensitivity	50 (7-93, 2/4)	100 (22-100, 2/2)	0.47
	Specificity	100 (100-100, 1155/1155)	100 (99-100, 1155/1157)	0.50
VDPV2	Sensitivity	70 (46-88, 14/20)	88 (62-98, 14/16)	0.26
	Specificity	100 (99-100, 1137/1139)	99 (99-100, 1137/1143)	0.29
Sabin 3	Sensitivity	75 (43-95, 9/12)	90 (55-100, 9/10)	0.59
	Specificity	100 (100-100, 1146/1147)	100 (99-100, 1146/1149)	0.62

Time taken to confirm poliovirus by VP1 sequence

During this study period, 27 samples with VDPV2 had the VP1 region sequenced using both diagnostic methods. Only samples of programmatic importance are sequenced following cell-culture (all serotype 2 viruses and any suspected vaccine-derived and wild-type polioviruses) whilst DDNS testing always yields a sequence for positive samples. For these 27 samples a median of six days were required between case onset and sample collection (range: 2-21 days) and a further median six days were required between sample collection and arrival of samples at the laboratory (range: 2-27 days). The time from receipt in the laboratory to a VP1 sequence took a median of 30 days (range: 21-41 days) via the standard algorithm, including a median of 8 days (range: 4-22 days) required for shipment between the virus isolation and sequencing lab, whilst DDNS was significantly quicker ($p < 0.001$, Mann–Whitney U test) requiring a median of seven days (range: 4-23 days) (see figure 1).

cVDPV2 outbreaks detected during the study period

During the study period, four cVDPV2 outbreaks occurred in the province of Maniema in the DRC and confirmed through routine testing (culture-ITD-sequencing). For two of the linages (RDC-MAN-3 and RDC-MAN-4) the sample confirming circulation (2nd case) was collected during the study period, whilst for RDC-MAN-2 the confirming sample was collected 42 days prior to the study period and processed during training. By the standard detection algorithm, the samples confirming these outbreaks required 27, 35 and 64 days respectively between sample collection and Sanger sequencing, a mean of 42 days. These same samples were processed in 6, 20 and 30 days respectively by DDNS, despite the samples for RDC-MAN-2 being collected prior to the study period, a mean of 23 days quicker. The fourth outbreak lineage, RDC-MAN-5, only had the first positive sample collected during the study period, but this sample similarly yielded a VP1 sequence 29 days earlier by DDNS. The geographic spread of cases identified by DDNS for the four outbreaks and relatedness of RDC-MAN-3 outbreak lineage is shown in figure 2. Based on the poliovirus VP1 molecular clock and these DDNS-derived VP1 sequences we estimate that the RDC-MAN-3

lineage emerged from a OPV2 vaccination campaign performed in the first quarter of 2020 (mean date 26.01.2020, 95% Highest Posterior Density 04.04.2019-16.09.2020.

Sequence comparisons

Where consensus cVDPV2 VP1 sequences were available from both DDNS and Sanger sequencing of culture isolate for the same sample, the similarity of the sequences was determined. The mean VP1 sequence identity comparing DDNS and the standard algorithm (including Sanger sequencing) for the 27 cVDPV2 with results for both methods was 99.98% (range: 99.60-100%). The absolute number of differences between sequences is shown in table 5.

Table 5 – Nucleotide differences in the VP1 region (903 base pairs) between sequences generated by Sanger sequencing of culture isolate and by DDNS from the same stool sample.

Number of nucleotide differences	Count (%)
0	25 (92.6%)
1	1 (3.7%)
2	0 (0%)
3	0 (0%)
4	1 (3.7%)
5+	0 (0%)

Costs and staff requirements

The DDNS assay consumable costs are approximately $20 per sample when performing multiplexed sequencing runs of 96 samples or $25 per sample when performing runs of 45 samples in lower throughput laboratories (see Supplemental Table 2). These figures include chloroform treatment, RNA extraction, nested PCR and nanopore sequencing. For chloroform treatment, cell-culture and qPCR alone, the cell-culture-based detection algorithm costs approximately $32 (see Supplemental Table 2), in addition to the cost of Sanger sequencing and shipping to the National Institute For Communicable Diseases (NICD) in South Africa where the sequencing is performed. Whilst some large items of equipment are required by both methods, DDNS avoids the requirements of microscopes, incubators, cell counters and tissue culture cabinets, whilst only needing the addition of a MinION or GridION sequencer (with MinIONs typically costing $1,000, including a sequencing reagents kit and a flow cell). Staff costs have not been included in the figures, yet the performance of DDNS at INRB required only five staff members; three laboratory scientists for RNA extraction, nested RT-PCR and nanopore sequencing and two bioinformaticians/data managers to perform data quality control and match the sequences to case data. Comparable steps from the cell-culture based algorithm requires four laboratory scientists for cell-

culture and qPCR, two support staff for maintaining the facilities and the support of additional sequencing staff and bioinformaticians at the NICD.

Discussion

Implementation of DDNS for detection of poliovirus in stool samples in DRC provided a rapid, sensitive and cost-effective tool for surveillance. In this study we have demonstrated the feasibility of implementation of this method in a national poliovirus laboratory and its performance alongside routine detection by the current cell-culture based algorithm.

Implementation of the method does not require a cell-culture facility or the transfer of samples to an overseas laboratory for Sanger sequencing, allowing all steps to be performed at one site in a single streamlined workflow. The per sample cost is at least $10 lower for high-throughput laboratories that can maximise the benefit of sample multiplexing during sequencing. This excludes further savings from international shipment of samples and sequencing at a centralised hub. Routine DDNS at INRB was implemented with contributions from five staff, compared to six for the cell-culture method. Moreover, it supported a workforce trained in molecular techniques including the preparation of sequencing libraries, performance of sequencing and the analysis of sequencing data. The skills and facilities required for DDNS can be rapidly redeployed to other pathogens during public health emergencies. With the global expansion of sequencing capacity there are increasing opportunities to foster the development of these skills and facilitate their contribution to disease surveillance and pathogen genomics, potentially through centralised bioinformatic support from either the GPLN, from sub-regional labs with expertise (e.g. INRB) or from other regional bodies (e.g. Africa CDC's Pathogen Genomics Initiative).

Implementation of the DDNS method has demonstrated that it can be performed significantly more quickly than cell-culture. DDNS VP1 sequences for VDPV2 positive samples were on average being generated a median 14 days after stool collection. This is similar to, but slightly slower than the 12 days we predicted when estimating the performance of DDNS based on stool sample collections from 2016–2020 [10]. These earlier estimates did not account for sample batching to maximise efficiency and minimise costs. With complete DDNS runs (96 samples including controls) being performed every 5.4 days, the effects of batching were however minimal, reflected in only a two day increase in the time to generate a sequence compared to our previous model-based estimates. Further improvements in speed could be achieved through automated RNA extraction or by decreasing the time taken to deliver samples to the laboratory, potentially even through the use of sample delivery drones given poor road conditions [16], or through the establishment of additional regional laboratories within DRC.

For samples required to confirm three of the four cVDPV2 lineages, DDNS generated the VP1 sequence required to initiate a response a mean of 23 days (range 6–30 days) quicker than culture. Earlier detection and response to outbreaks leads to few cases and a higher probability of truncating ongoing transmission [2,10]. During this research study, sequences were not used to inform outbreak response because the method has not yet been accepted or recommended by the Global Polio Laboratory Network

(GPLN)[17]. However, we are working to meet the requirements for GPLN recommendation, including pilot implementation of DDNS in additional laboratories worldwide.

Despite the lower raw read sequencing accuracy of nanopore compared to Sanger sequencing, the generation of consensus sequences gave a mean similarity of 99.98%. The sequence identity was < 100% for just 2/27 samples, which may be addressed by further improvements to nanopore chemistry and the analytical software, for example through the more accurate resolution of homopolymers. Where a relatively large difference remains (the sample with 4 nucleotide differences between consensus sequences from DDNS and Sanger sequencing) this may represent different viral populations within the gut of the AFP case. This case had a pair of stool samples collected a day apart, and the Sanger sequences for the pair also differed by one nucleotide. Through removal of competitive viral cell-culture and with the use of next-generation sequencing, DDNS does however allow the identification of multiple viral templates from a single sample, as demonstrated by the detection of both Sabin 1 and Sabin 3 in two of the samples. Improved calling of haplotypes could even allow the resolution of very closely related viral populations (differing by only one or two nucleotides) from within a single sample.

Although not statistically powered for a comparison to culture, the study would indicate that DDNS is at least as sensitive as culture for the detection of poliovirus. The additional poliovirus detections by DDNS are not suggestive of contamination given that they tended to occur either across sample pairs from the same AFP case or in sample pairs where culture tested positive for one of the pair. Furthermore, the protocol allows for rapid identification of contamination for viruses of programmatic importance (wild-type and VDPVs) because of the rapid evolution of poliovirus and the low likelihood of identical VP1 sequences apart from for those samples collected from the same case or their contact. A quality assurance program for DDNS has been developed, including the use of a lyophilised virus positive control, and quality control flags built into bespoke software now developed for DDNS (PIRANHA; https://github.com/polio-nanopore/piranha).

An additional method of direct detection by qPCR[18] (without sequencing) is being evaluated by the GPEI and a comparison between this method and DDNS has not yet been made. Further evaluation is required to compare accuracy of detection and the speed at which a VP1 sequence can be generated by the two methods, along with consideration of ease of implementation and staff training requirements.

An advantage of DDNS is the potential to completely replace cell culture in most polio laboratories, which is both costly and undesirable as poliovirus goes in to global containment [19]. For DDNS to be sustained, challenges with supply chains must be overcome, as countries likely to benefit most from rapid detection are also likely to be more difficult to supply with reagents for nanopore sequencing. For laboratories that also test environmental surveillance (ES) samples, DDNS can be used for these samples, providing sequencing reads for multiple virus templates, as may occur in sewage [13]. However, direct detection methods typically only allow relatively small sampling volumes (hundreds of microlitres for an RNA extraction as opposed to 4mL for the eight cell-culture flasks now employed), hence greater concentration of ES samples and/or large volume RNA extractions will be required to allow achieving a similar or

greater degree of detection sensitivity. We are currently optimising these methods for ES samples, which show considerable promise [20].

With the possibility of rapidly generating sequences within the country of sample origin, a next logical step is the local analysis of these data to provide actionable information. Mutation within the poliovirus VP1 region following a molecular clock [21], hence variation within sequences can allow the construction of phylogenetic trees and the estimation of dates of outbreak emergence, as demonstrated in this manuscript. It is also possible to reconstruct viral spread, identifying geographic regions that are sources and sinks of the virus. Combined with the rapid generation of sequencing data, such analyses could facilitate agile and accurately targeted responses to viral outbreaks.

Online Methods

Sample Collection

Stool samples were collected during routine AFP surveillance in DRC between 10th August 2021 and 4th February 2022. All 2339 samples received at the national polio laboratory (INRB) from AFP cases, the community and contacts were processed in this study. It is recommended that two stool samples are collected from children aged 0-14 years old with AFP, within 14 days of onset of paralysis and at least 24 hours apart. Single stool samples from healthy contacts of children with AFP are additionally typically collected from children aged <5 years old and occasionally from the wider community.

Sample processing

Chloroform-treated stool supernatant (as described in [22]) underwent RNA extraction using either Roche High Pure Viral RNA Kits (#11858882001) or QIAamp Viral RNA Mini Kits (#51106) according to manufacturers' protocols using a volume of 200 µL and 140 µL of supernatant respectively. Extracted RNA was stored at + 4 ° C during the preparation of the nested PCR if performed on the same day or – 80 ° C if delayed more than 24 hours.

Direct detection and nanopore sequencing (DDNS)

DDNS based on a nested, barcoded PCR and amplicon sequencing on nanopore sequencers was performed as described in [13]. In brief, a nested PCR was performed using 5 µL of extracted RNA and pan-Enterovirus primers [23] with the product used for a poliovirus specific VP1 PCR using barcoded primers [13]. 2 µL of each PCR product was pooled prior to cleaning with AMPure XP beads (Beckman Coulter, #A63880) and the sequencing library prepared with Oxford Nanopore Technologies (ONT) LSK-110 ligation sequencing kits. The complete protocol can be found at https://www.protocols.io/workspaces/poliovirus-sequencing-consortium.

Libraries were sequenced on the ONT GridION, MK1c or MinION sequencers for between four and twelve hours using R9.4 flow cells. Sequencing runs were performed between 29th September 2021 and 17th

February 2022.

Bioinformatics

Sequence basecalling was performed using guppy with demultiplexing and mapping of the reads performed using RAMPART (https://github.com/artic-network/rampart) [24] and the realtime-polio analysis module (https://github.com/polio-nanopore/realtime-polio). VP1 consensus sequences were generated by four iterative rounds of mapping using the mafft algorithm[25] and polishing with racon[26] prior to consensus calling with medaka[27]. The dated maximum-likelihood tree for the RDC-MAN-3 outbreak was created in R version 4.1.3 using the Analyses of Phylogenetics and Evolution (ape) package [28], Phylogenetic Reconstruction and Analysis (phangorn) package [29], and the treedater package [30]. A molecular clock rate of 0.01 substitutions per site per year was assumed based on Jorba *et al,* 2008 [31].

Statistical Analysis

Time taken between case onset and a sequence being generated via DDNS and the standard algorithm were compared using Mann–Whitney U test. Exact binomial confidence intervals were calculated for the sensitivity and specificity of the two methods and comparison made using Fisher's exact test.

Declarations

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Figures

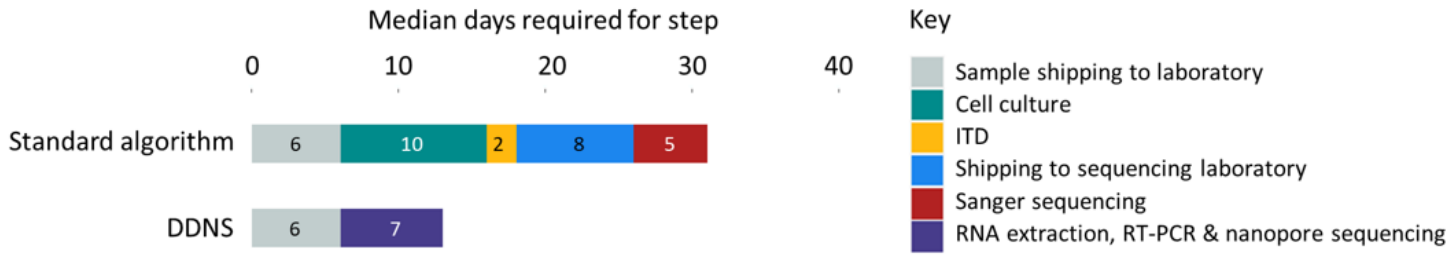

Figure 1

Median time required for each diagnostic step in the two protocols for 27 cVDPV2 positive stool samples.

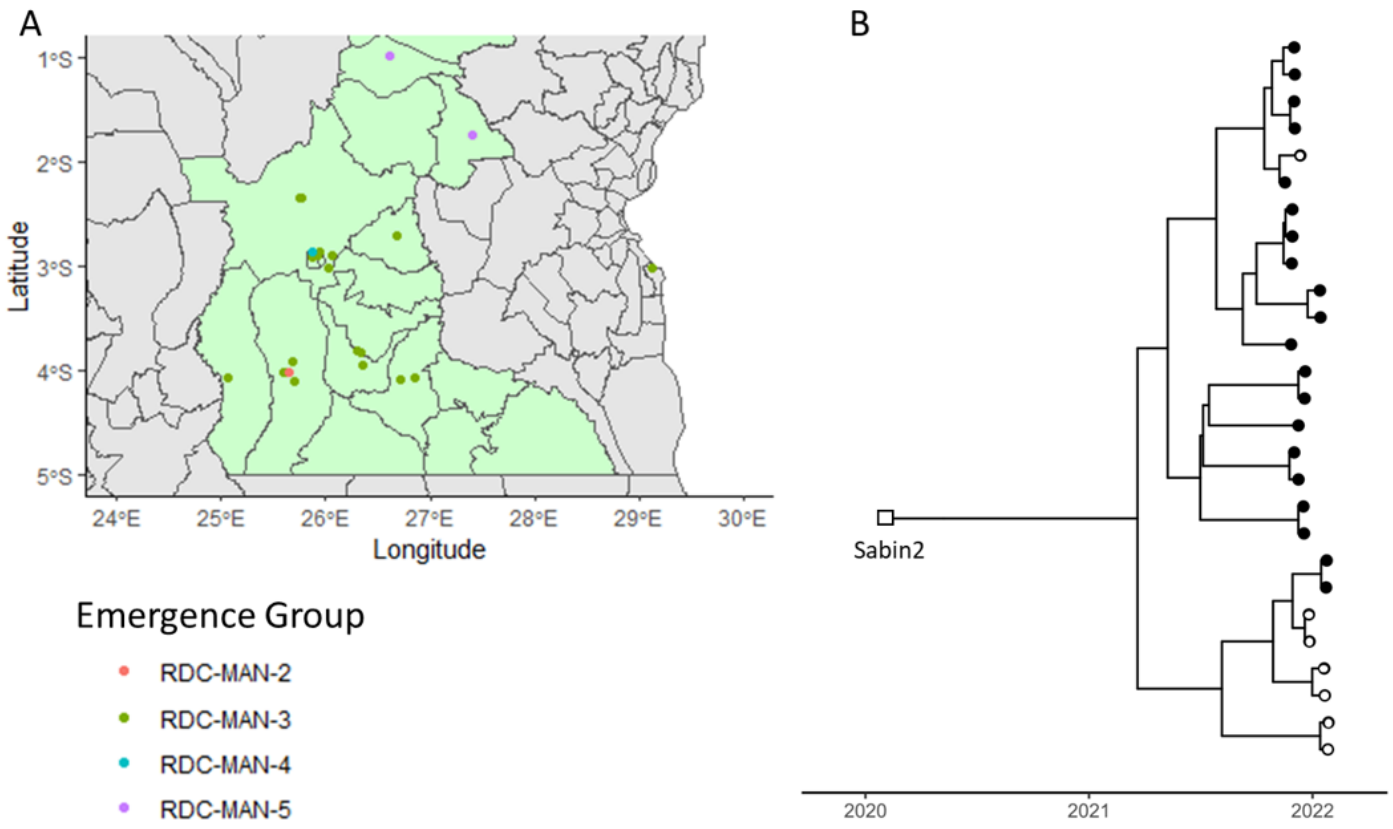

Figure 2

cVDPV2 outbreaks detected by DDNS. A) Cases from the four Maniema lineages detected during the study period (Maniema province highlighted in green). Cases are plotted by district, with placement within the district determined at random. B) Tip-dated phylogenetic tree showing the maximum likelihood emergence date of RDC-Man-3 lineage and its subsequent diversification over time. Solid tips indicate that the sample that the DDNS detection was matched by a cell-culture-based detection of a cVDPV2 from the same sample. Cases confirmed by Sanger sequencing but without a corresponding DDNS sequence (n = 2) were not included in the analysis.

Supplementary Files

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- SupplementalMaterial.docx