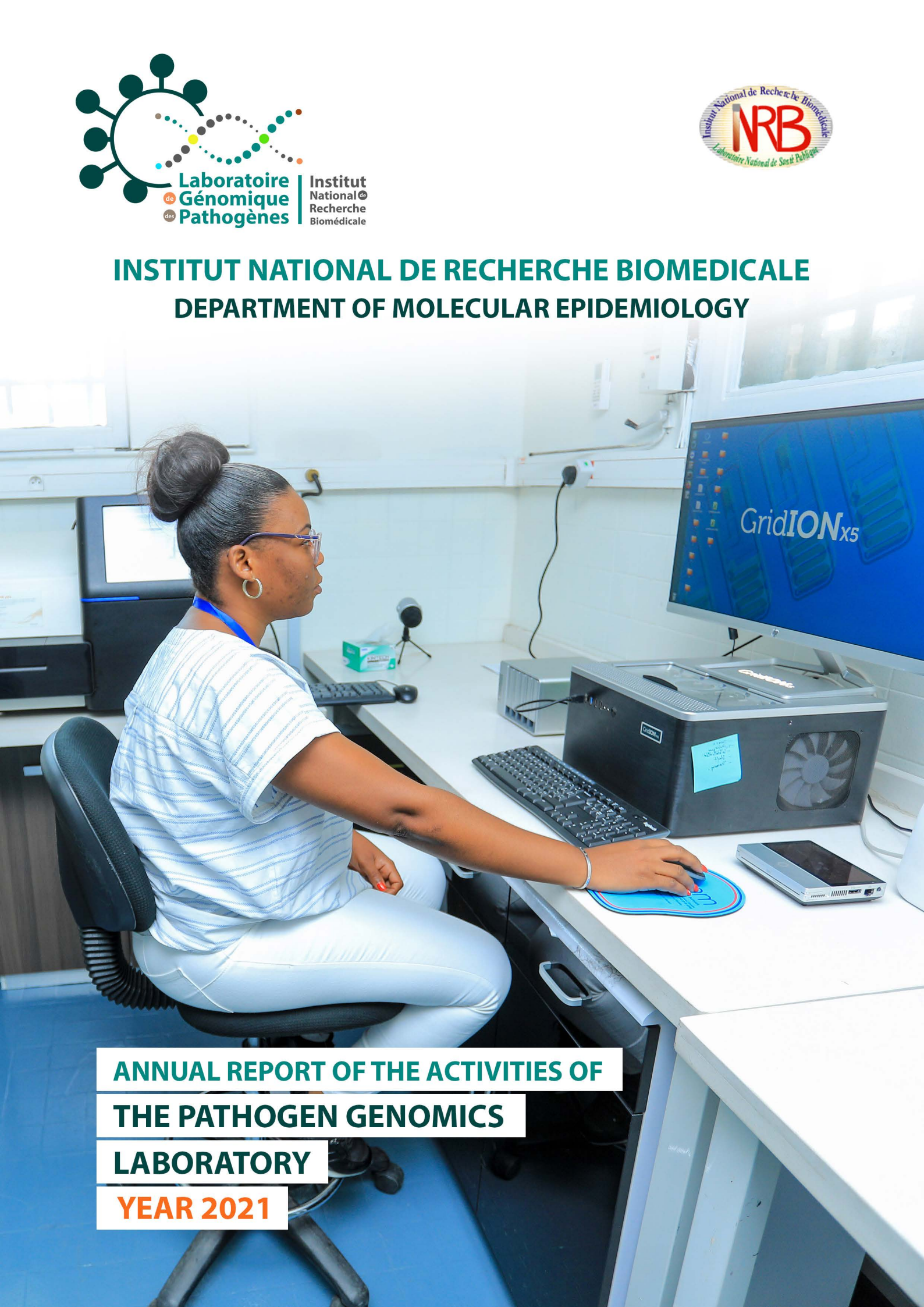
Laboratoire de Génomique des Pathogènes | Institut National de Recherche Biomédicale

INSTITUT NATIONAL DE RECHERCHE BIOMEDICALE

DEPARTMENT OF MOLECULAR EPIDEMIOLOGY



ANNUAL REPORT OF THE ACTIVITIES OF THE PATHOGEN GENOMICS LABORATORY

YEAR 2021

Table of contents

List of Abbreviations

BMGF : Bill and Melinda Gate Foundation

CAR : Central African Republic

cVDPV : circulating Vaccine-Derived Polioviruses

DDNS : Directive Detective Nanopore Sequencing

DNA : Deoxyribonucleic acid

DRC : Democratic Republic of Congo

EVD : Ebola virus disease

INRB : National Institute for Biomedical Research

NGS : Next Generation Sequencing

PCR : Polymerase Chain Reaction

PVS : Poliovirus Sauvage (Wild Poliovirus)

RNA : Ribonucleic Acid

ONT : Oxford Nanopore Technology

VOC : Variant Of Concern

WHO : World Health Organisation

List of Tables and Figures

1. Tables

2. Figures

I. Introduction

Since 2018, the National Institute of Biomedical Research has acquired equipments, reagents and consumables made it possible to carry out NGS analyzes in the country via the Illumina and Oxford Nanopore Technologies platforms (ONT).

The various training received have enabled the team sequencing of pathogens to appropriate the technology. This achievement has been an important support for the following activities:

- Confirmation of the last two outbreaks of the Ebola Virus Disease (12th and 13th);
- 3 additional investigations around the 10th Ebola outbreak in the DRC:
  - The impact of the V75A mutation,
  - The bacteriological and virological profile of negative samples collected during the outbreak crumb.
  - The link with the 12th and 13th outbreaks.
- Detection of variants of concern (VOC) of Covid-19 circulating in Central Africa.
- Direct molecular detection of Polio virus with the Nanopore platform.
- Confirmation of the Monkeypox outbreak in Maniema
- Confirmation of the plague outbreak in the province of Ituri.

All these activities took place mainly in Kinshasa where sequencing is implemented in its demanding "wet lab" and "dry lab" or bioinformatics. The same approach has also been successfully undertaken in the North – Kivu, precisely in Goma.

II. Overview of the Sequencing Methodology

Throughout the year, several approaches and protocols were used for sequencing according to the objective for follow-up. Note that amplicon sequencing was the strategy applied for the sequencing of Ebola, Polio, and SARS-CoV-2, while the metagenomic approach has used for the detection of Monkeypox and Yersinia pestis. As far as bioinformatics analyzes are concerned, the iVar and artic pipelines were used for sequencing by amplicons respectively in the Illumina and Nanopore. The consensus fasta pipeline and PathDisco have been used for metagenomic analyzes.

III. Activities performed

1. Ebola Virus Desease (EVD)

a. . Confirmation of the last two (12th and 13th) EVD outbreaks

Two outbreaks were declared this year in North Kivu: in Butembo in February 2021 and in Beni towards the end of October 2021. Their situation is summarized in the table below.

	12th outbreak	13th outbreak
Health area	Biena (Butembo Extension)	Béni
Period	From February 7 to May 3, 2021	From October 07 to December 16, 2021
Total samples tested	1758	1175
Number of patients tested positive	11	8
Number of samples sent to Kinshasa for sequencing	11	1
Number of positive samples sequenced in the field	0	7 including 6 complete genomes
Additional investigations in Kinshasa	Link with the samples of Butembo	Link with Butsili sample Sequencing of 30 samples of the 10th epidemic in probable link with the index case... the analyses continue

Table 1 : Situation of the 2 outbreaks that occurred in North Kivu this year

For the 2 re-emergences, the new cases detected and sequenced have a link with a group of samples from the 10th outbreak (Ituri variant). It was therefore not a question of a new virus introduction (spillover), but rather a resurgence probably from a survivor. For the Biena outbreak, the patient had 5 mutations of more than the closest genome, revealing a rate of substitution slowed and characteristic of the infection prove- from a persistent source. The same suggestion of infection from a persistent source was also issued for the Butsili outbreak (Béni) following the reduction of 5.6 times of the evolutionary rate. The new genome (21-BEN114) had six divergent mutations of the closest genome, which was collected in July 2019[1].

1. https://virological.org/t/oct-2021-evd-case-in-drc-linked-to-2018-2020-nord-kivu-evd-outbreak/762

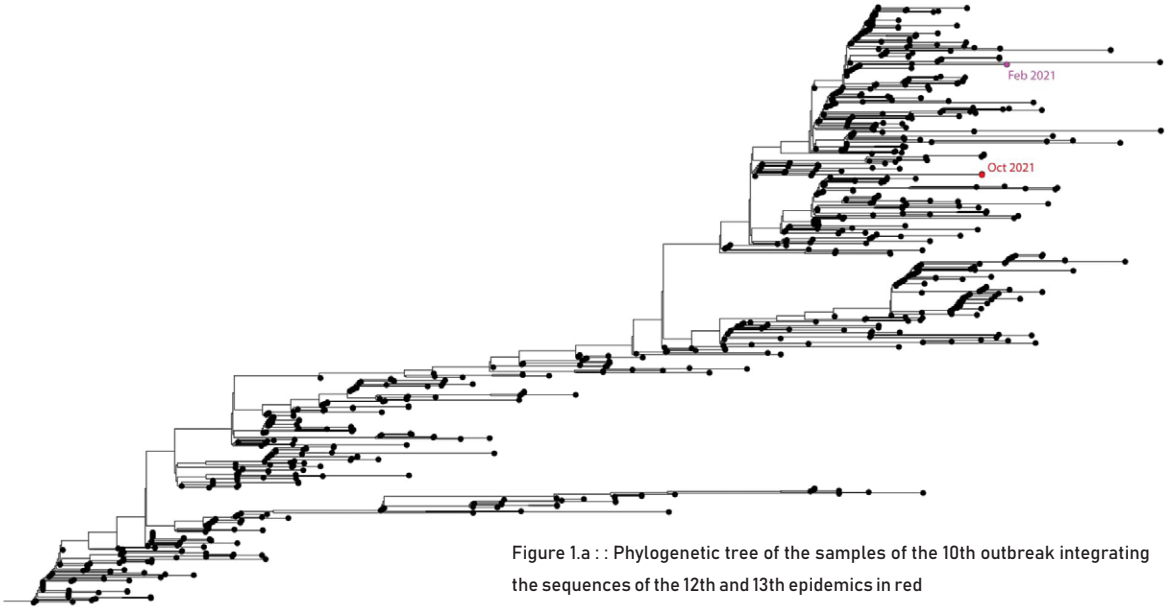


Figure 1.a : : Phylogenetic tree of the samples of the 10th outbreak integrating the sequences of the 12th and 13th epidemics in red

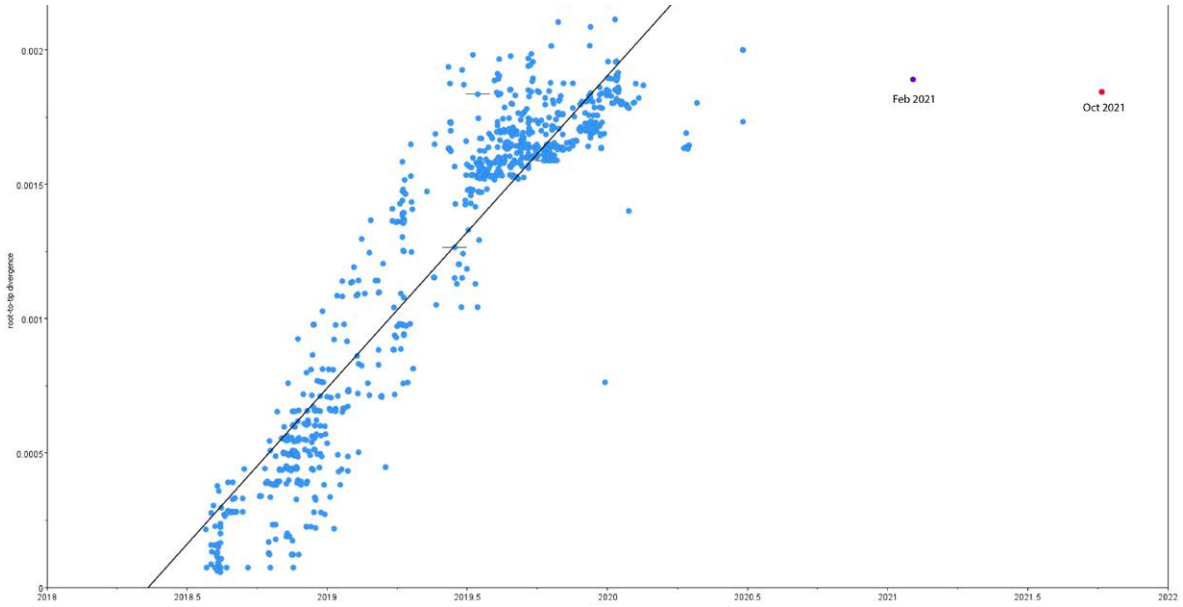


Figure 1.b : Illustration of the divergence of samples from the 12th and 13th outbreaks on this time clock

b. Additional investigations around the 10th Ebola outbreak

i. The link with the 12th and 13th outbreaks

After confirmation of the outbreaks, sequencing enabled set to establish links with genomes isolated during the 10th epidemic. For the 13th outbreak, the closest relatives (BEN24348 and KAT7440) were isolated from samples taken from deceased patients. They couldn't therefore not be the source of the infection, but rather probably in the same chain of transmission or close to of the persistently infected survivor.

Furthermore, the first diagnosed case (21-BEN114) does not appear to be the source case according to epidemiological reports. Indeed, it followed a group of three deaths EVD suspects reported by area officials health events in Beni on September 30 which occurred in a household in the Butsili health zone on 14, 19 and 29 September 2021. The three suspected EVD cases were living in the same neighborhood as the confirmed case. No sample has been taken in these cases and safe and dignified burials have not been performed. Thus, it was hypothesized that which one or more of the six mutations acquired for- may have occurred during human-to-human transmission normal before sample collection of 21-BEN114. This gave rise to additional investigations having consisted of research in databases as well as than in the narratives of epidemiological investigations, all samples from the 10th outbreak, from Butsili and Kanzulinzuli health areas sampled around from July 2019.

30 samples were found and sequenced (see figure on the next page). Even new genomes (in grey) are between BEN24348 and the 21-BEN samples (in green). The patient INRB-002269 (Lab ID BEN_24419 and Epi ID BENV_25508) shares a mutation with the branch to the 21-BEN samples, but also has additional mutations. He is also a contact of the index cases and was recently sampled in Beni. Serum from these contacts was negative for Ebola (Genexpert) and was sent to INRB for sero-

logy (IgM and IgG) with other contacts. A report on the serology results will follow shortly. Patient INRB-002269 (Lab ID BEN_24419 and Epi ID BENV_25508) additionally tested positive for IgG and negative for IgM (despite a low signal). Sequencing of all cases related to INRB-002269 could be useful.

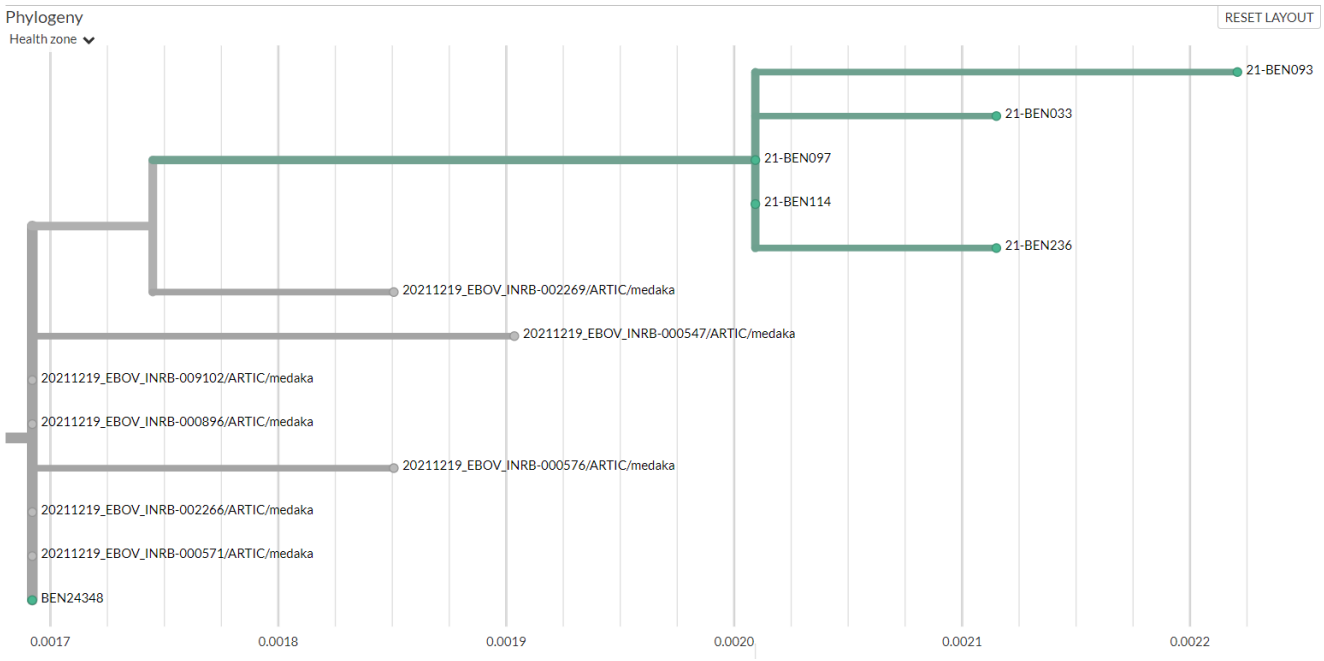


Figure 2 : Screenshot of the phylogenetic tree integrating the samples of the 13th outbreak and 7 of the 30 most genetically related sequenced samples

ii. The impact of the V75A mutation

The 10th outbreak of EVD that has plagued the DRC is the 2nd in the world both in terms of its duration, 22 months, and its importance in terms of recorded cases. Declared on August 1, 2018 in the province of North Kivu, it has spread to the provinces of Ituri and South Kivu, with cases exported to Uganda. This outbreak presented particular challenges because it occurred in an area of active conflict. There have been 3,470 cases, 2,287 deaths, and 1,171 survivors up to June 25, 2020. More than 800 samples distributed throughout the outbreak have been sequenced to date. A non-synonymous mutation in the GP protein, V75A, has been observed (Figure 4).

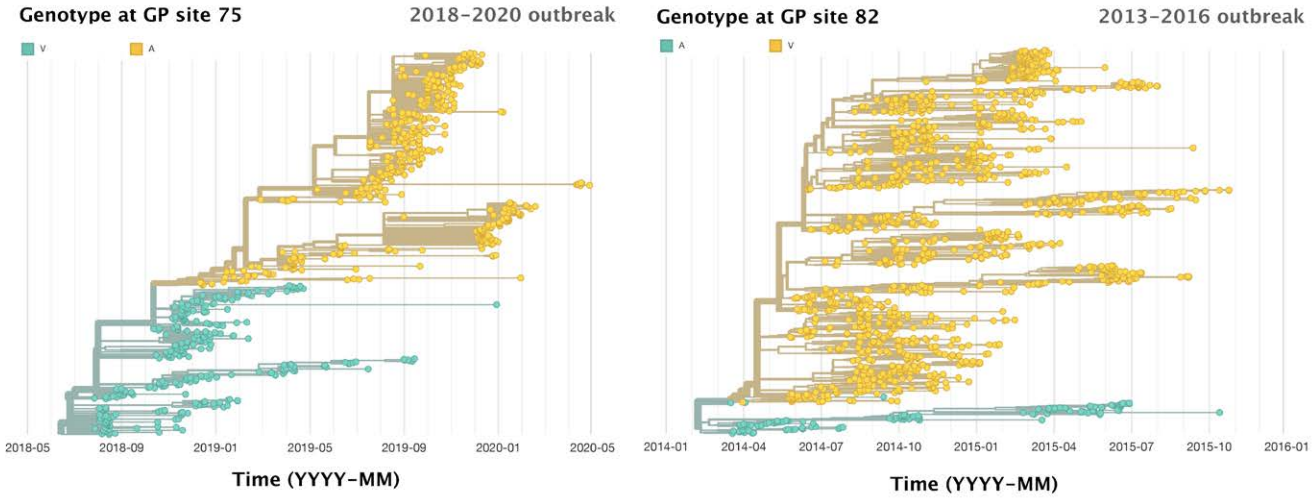


Figure 3 : Screenshot on Nextstrain showing the phylogenetic tree of samples sequenced during the 10th outbreak and the introduction of the V75A mutation and those of the West African outbreak for the A82V mutation

For the Biena outbreak, the patient had 5 more mutations than the nearest genome, indicative of a slowed substitution rate and characteristic of infection from a persistent source. The same suggestion of persistent source infection.

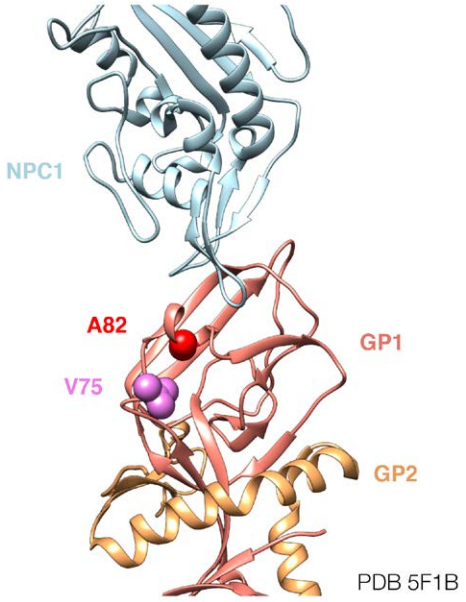


Figure 4 : A82V mutation found in West Africa and having the same pattern as V75A

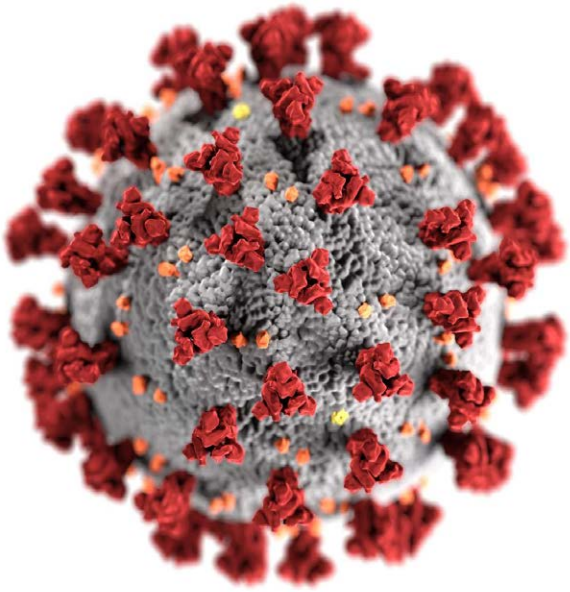
The same question arose for the V75A mutation, which has become fixed. The investigations consist in sequencing as many samples as possible and above all covering the supposed period of the mutation's appearance. This will allow us to compare patients with the mutation to those without it and to determine the impact on the evolution of the outbreak in terms of infectiousness, transmissibility, lethality, vaccine escape or experimental treatment.

iii. Bacteriological and virological profile of negative samples 10th outbreak

During the 22 months of the 10th outbreak, the response performed tests for more than 220,000 samples. However, only those from nearly 3470 cases (just over 20,000 samples) returned positive for Ebola PCR. Of the samples that came back negative, a smaller proportion belonged to asymptomatic contact cases undergoing follow-up, and the majority (more than 80%) were taken from symptomatic patients, meeting the definition of cases during the outbreak. For this great majority of patients, investigations by metagenomic approach of the negative samples of the 10th outbreak are in progress. Their objective is to determine the bacteriological and virological profiles.

A pilot study has been launched on 274 samples. 96 libraries have already been sequenced and are undergoing bioinformatics analysis.

2. Covid-19 pandemic



a. Introduction

In the DRC, the first Covid-19 case was diagnosed on March 10, 2020 and the DRC has recorded up to December 31 nearly 79632 confirmed cases including 2 probable cases and 1135 deaths. The first sequence was published on the Gisaid platform 2 weeks later, on 25 March 2020. The DRC was among the first countries in the world to publicly share its sequences on Gisaid in order to guide the global response.

Adhering to the Pathogen Genomics Initiative (PGI), since February 2021, the National Institute of Biomedical Research (INRB) has been designated by WHO and Africa CDC as the Regional Reference Laboratory for the sequencing of SARS-Cov-2 samples from Chad, the Republic of Congo, Cameroon, and the Central African Republic, allowing the study of viral mutations in time and space that can also help track the spread of the pathogen and better understand potential transmission routes and transmission dynamics.
With the support and efforts of several partners, the country was able to sequence the samples received in 2021, the year marked by the 3rd and 4th waves.

b. Activities performed

The activities carried out during the year 2021 consisted of:

- Receipt and inventory of samples;
- Sequencing ;
- And Training.

i. Receipt and inventory of samples

In addition to the DRC samples, the sequencing laboratory analyzed samples from 4 other Central African countries, such as Cameroon, Chad, Central African Republic, Republic of Congo (Table 1).

For DRC, samples were collected from at least 7 of the 26 provinces (Table 2).

All positive specimens should have good viral loads (Ct<30 for at least one of the targets, those below 28 being preferred), before being sent to the sequencing laboratory. Samples from the DRC were received in collaboration with the influenza and respiratory virus laboratory of INRB, the University of Kinshasa, selected hospitals in Kinshasa and provincial divisions. Details are provided in the table below.

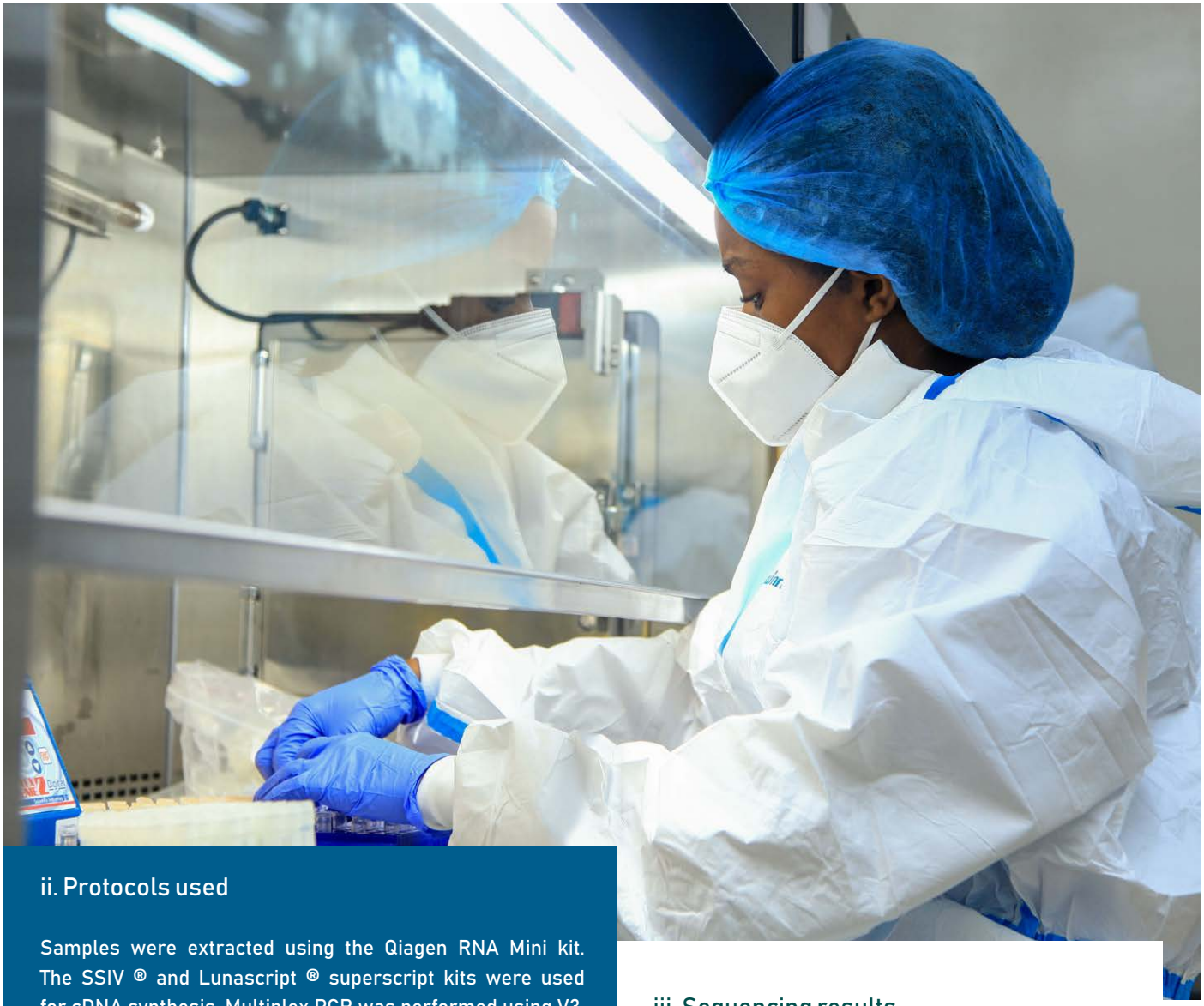
Country	Number of shipments	Number of samples	%
Cameroun	2	588	22.8
Chad	3	60	5.5
Central African Republic	3	141	5.5
Republic of Congo	5	486	18.9
Democratic Republic of Congo	NA	1302	50.5
Total		2577	100

Table 2 : Samples received by country

Province	Coverage over 80%	Total
Haut Katanga	20	50
Haut Uélé	14	23
Kinshasa	502	682
Kongo Central	6	13
Lualaba	9	18
Nord Kivu	18	24
Tshopo	2	5
Not specified	122	190

Table 3 : Number of DRC samples received by province

These samples were sent to the biobank for long-term storage.



ii. Protocols used

Samples were extracted using the Qiagen RNA Mini kit. The SSIV ® and Lunascript ® superscript kits were used for cDNA synthesis. Multiplex PCR was performed using V3, V4, Midnight or Qiagen primers. For the preparation of the libraries, several protocols and kits were used in the 2 Illumina and Nanopore NGS platforms: Rapid Barcoding Kit (Midnigth), SQ-LSK109, Nextera DNA Flex, Qiaseq. The artic or iVar pipelines were used for bioinformatics analysis. The obtained consensus was submitted to Pangolin and/or Nextclade software for variant assignment before being published on Gisaid.

iii. Sequencing results
a. DRC

The DRC had 2 waves this year and the team analyzed over 1005 samples of which 693 had a sequenced genome coverage of over 80% (Table 3). Performance varied throughout the year and was mainly a function of the quality of the samples received.

Sequencer	Platform	Cover>80%	Grand Total
MiSeq	Illumina	366	583
MinION	ONT	36	362
GridION	ONT	281	15
Mk1C	ONT	10	45

Table 4 . Number of samples per platform and sequencer

Figure 5 : Central African countries covered and supported by INRB in genomic surveillance

For the DRC, the variants detected are illustrated in the figure below. We note that the Delta variant circulated throughout the year before predominating during the 3rd wave. It successively gave way to the B.1.640 and Omicron variants.

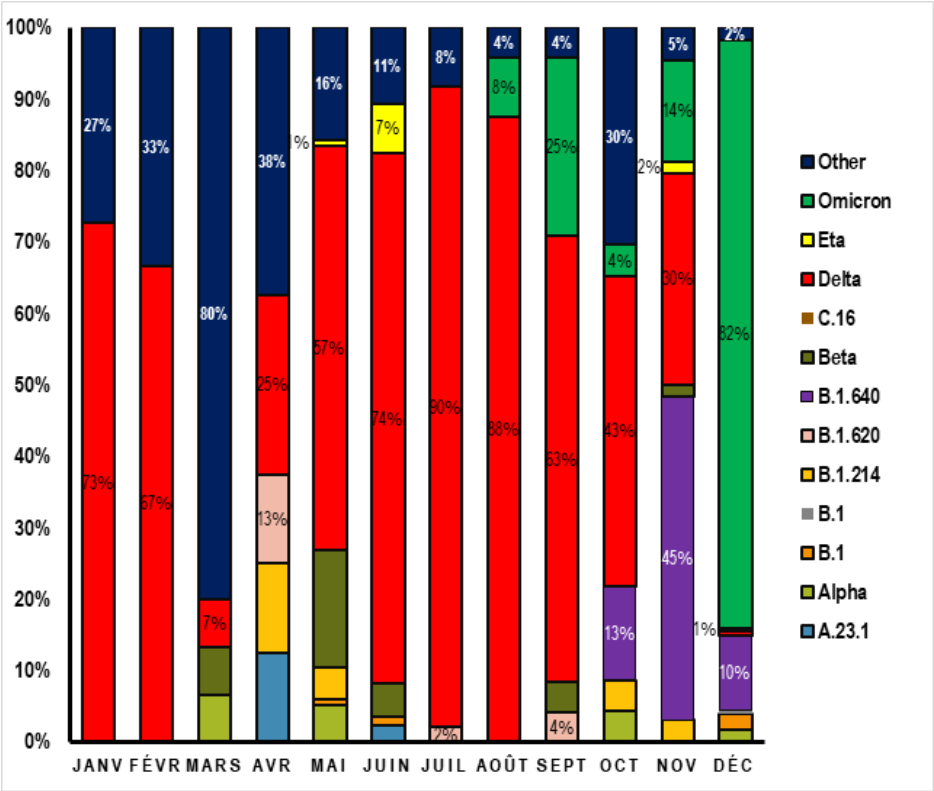


Figure 6 : Variants detected in 2021

b. Republic of Congo

From Congo, there were 5 shipments, 3 of which were in the last 2 months. Samples from the Republic of Congo were dominated by Alpha (June shipments), B.1.620 (October), B.1.640 (end of November), Delta and Omicron, respectively.

1st shipment: 100 samples (06/15/21)

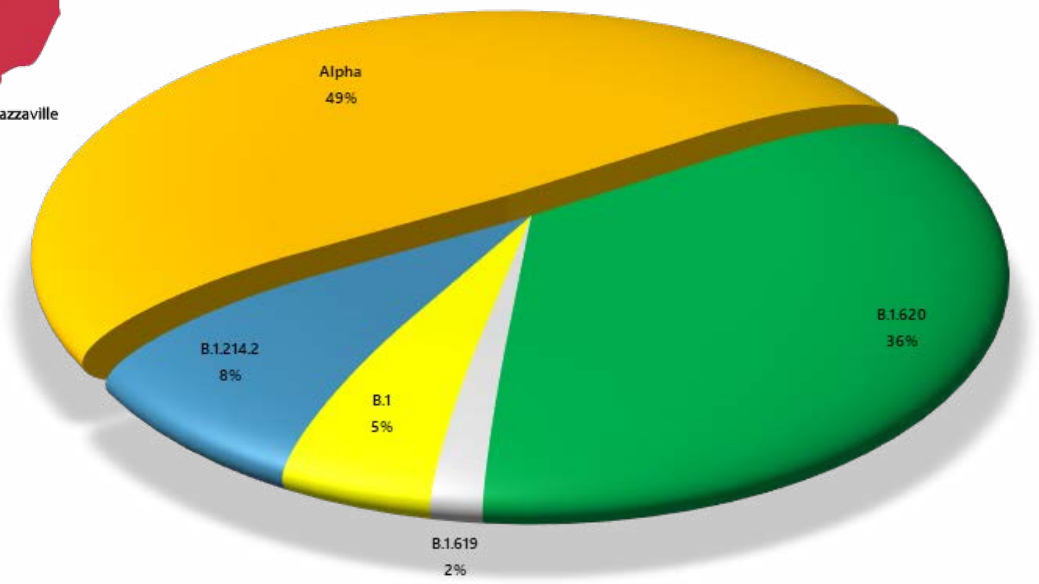


Figure 7 : Distribution of variants detected in the samples of the 1st shipment from the Republic of Congo

2nd shipment: 73 samples (10/20/21)

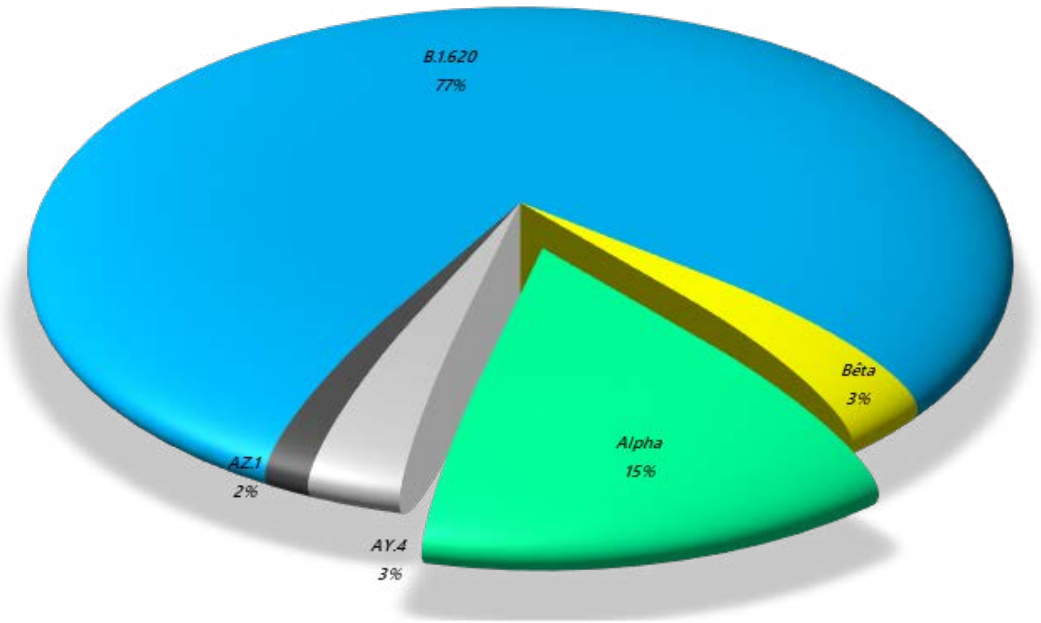


Figure 8 : Distribution of variants detected in samples from the 2nd shipment from the Republic of Congo

3rd shipment: 160 samples (11/23/21)

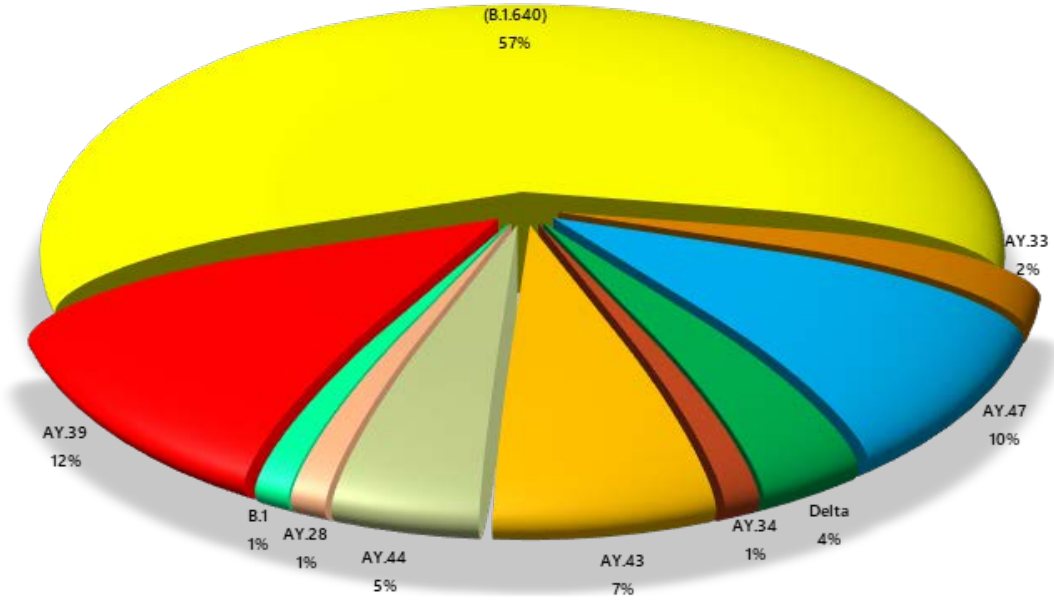


Figure 9: Distribution of variants detected in the samples of the 3rd shipment from the Republic of Congo

4th shipment: 81 samples (12/12/21)

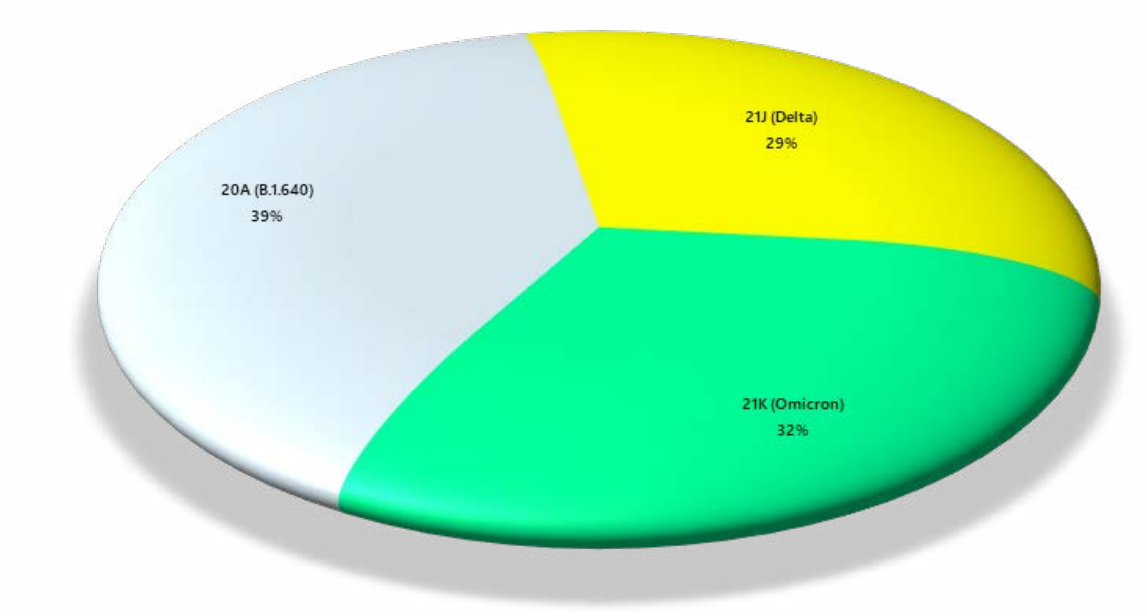


Figure 10 : Distribution of variants detected in the samples of the 4th shipment from the Republic of Congo

5th shipment: 72 samples (12/18/21)

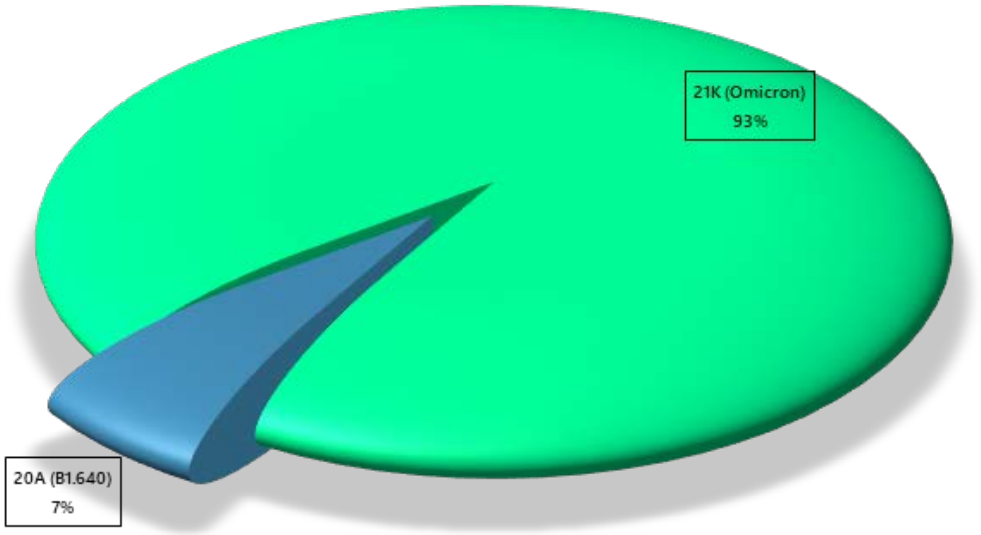


Figure 11 : Distribution of variants detected in the samples of the 5th expedition from the Republic of Congo

c. Chad

For Chad, there were 3 shipments dominated respectively by the variants B.1.1 (July 2021 shipment) and Delta (2nd and 3rd shipments).

1st shipment: 20 samples (07/13/21)

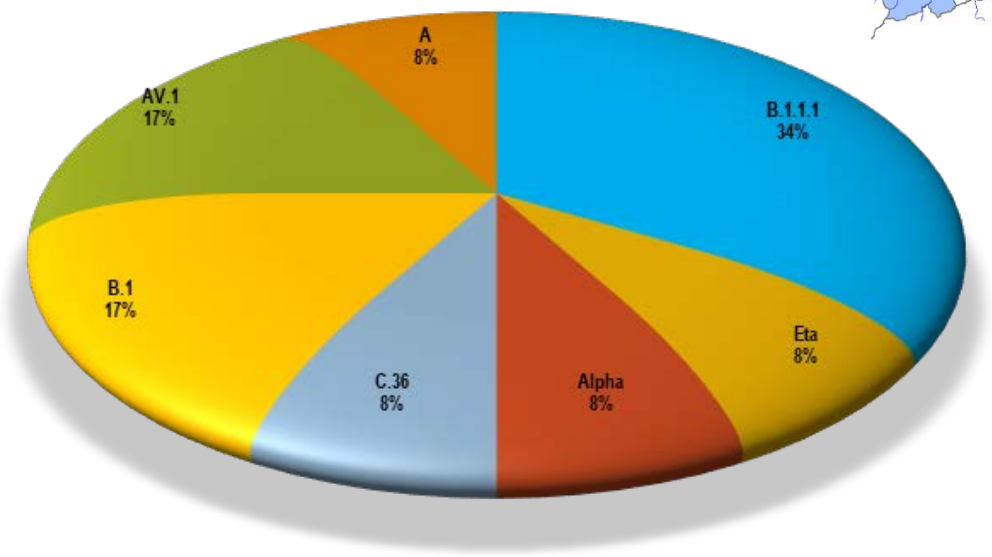


Figure 12 : Distribution of variants detected in the samples of the 1st expedition from Chad

2nd shipment: 20 samples (09/27/21)

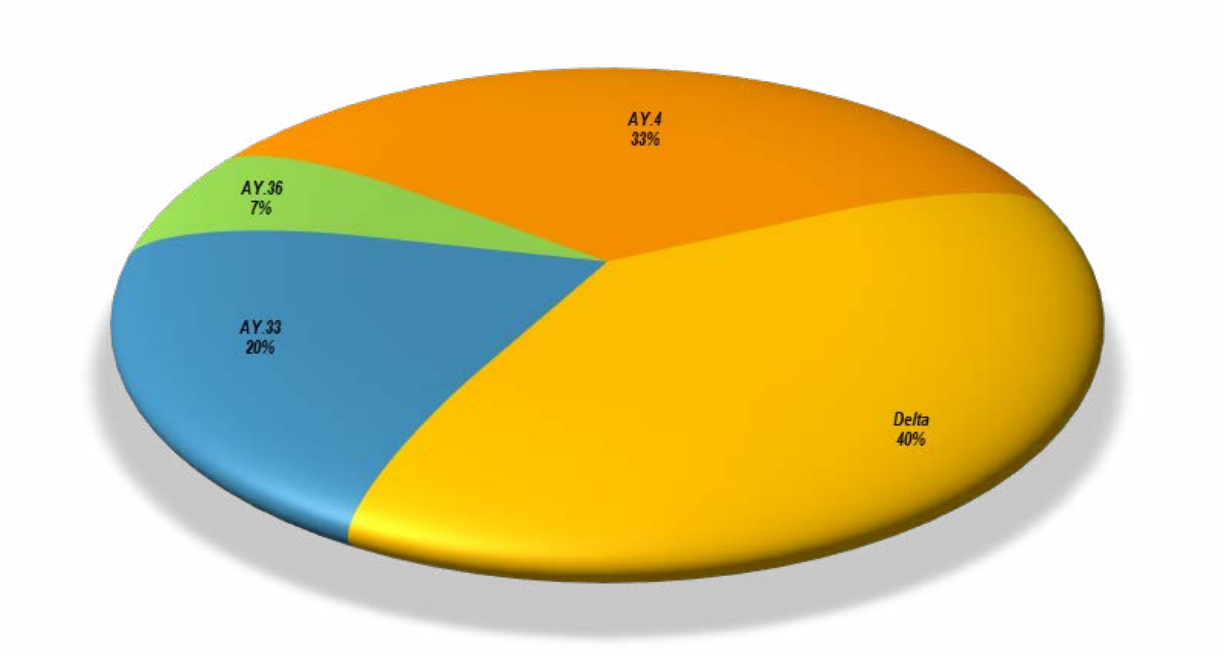


Figure 13 :Distribution of variants detected in the samples of the 2nd expedition from Chad

3rd shipment: 20 samples (11/24/21)

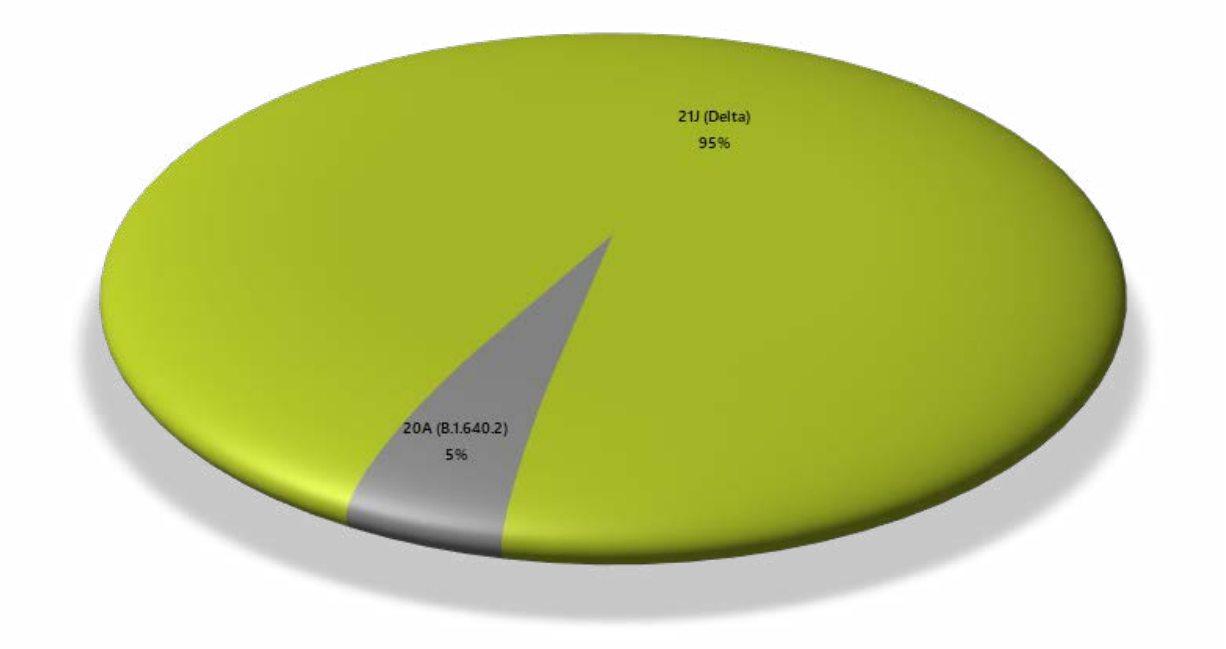


Figure 14 : Distribution of variants detected in the samples of the 3rd Chad expedition

c. Central African Republic

Among the CAR samples, the majority variants by shipment are B.1.177, Delta variant and B.1.640, respectively.

1st shipment: 50 samples (04/12/21)

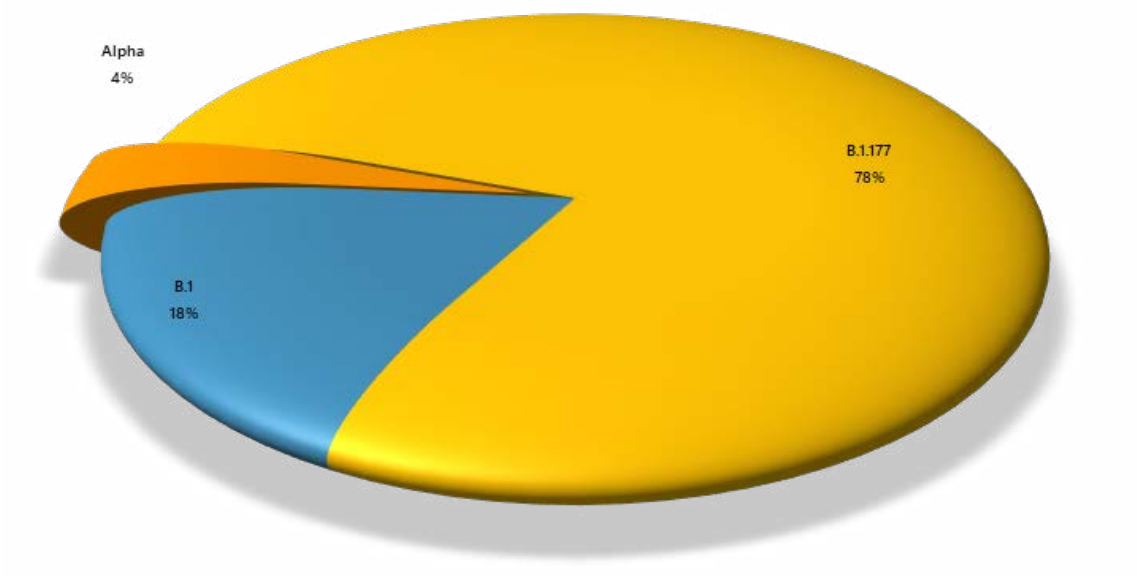


Figure 15 : Distribution of variants detected in the samples of the 1st CAR shipment

2nd shipment: 51 samples (08/07/21)

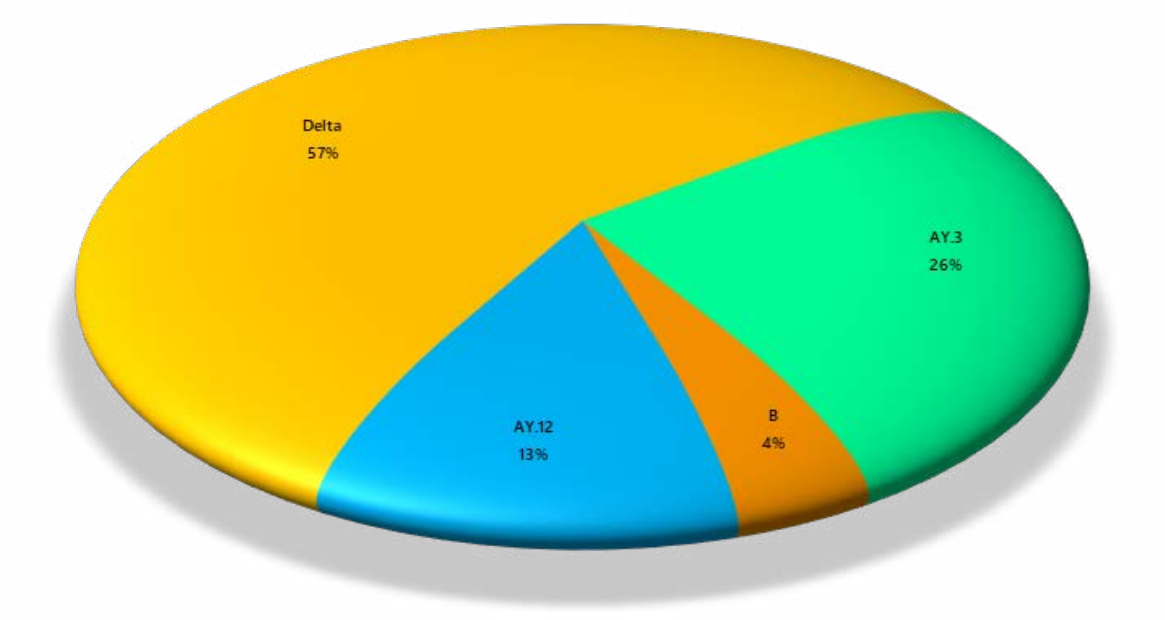


Figure 16 : Distribution of variants detected in the samples of the 2nd CAR shipment

3rd shipment: 50 samples (12/07/21)

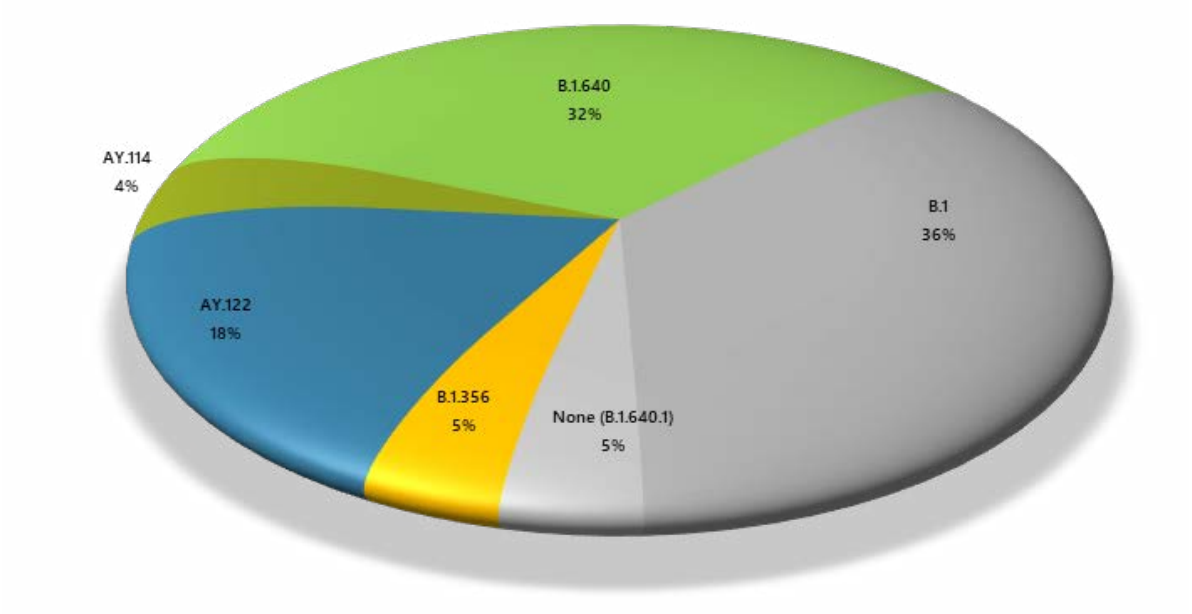
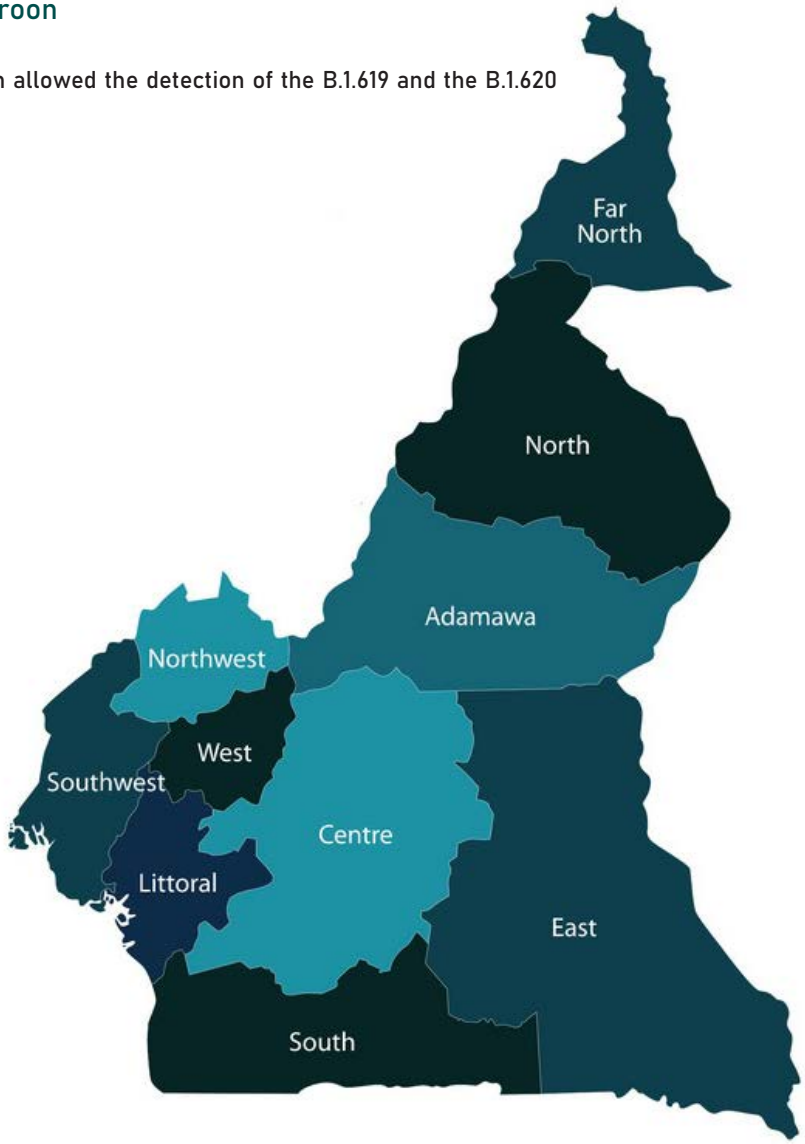


Figure 17 : Distribution of variants detected in the samples of the 3rd CAR shipment

d. Cameroon

Cameroon allowed the detection of the B.1.619 and the B.1.620 variants.



1st shipment: 100 samples (04/29/21)

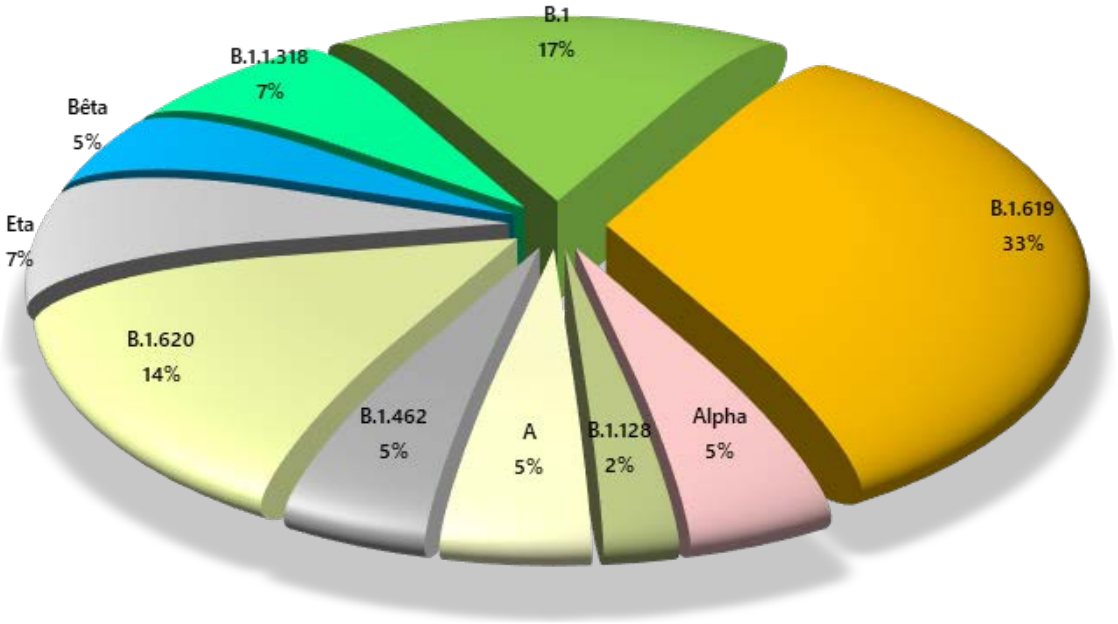


Figure 18 : Distribution of variants detected in the samples of the 1st expedition Cameroon

2nd shipment: 488 samples (08/23/21)

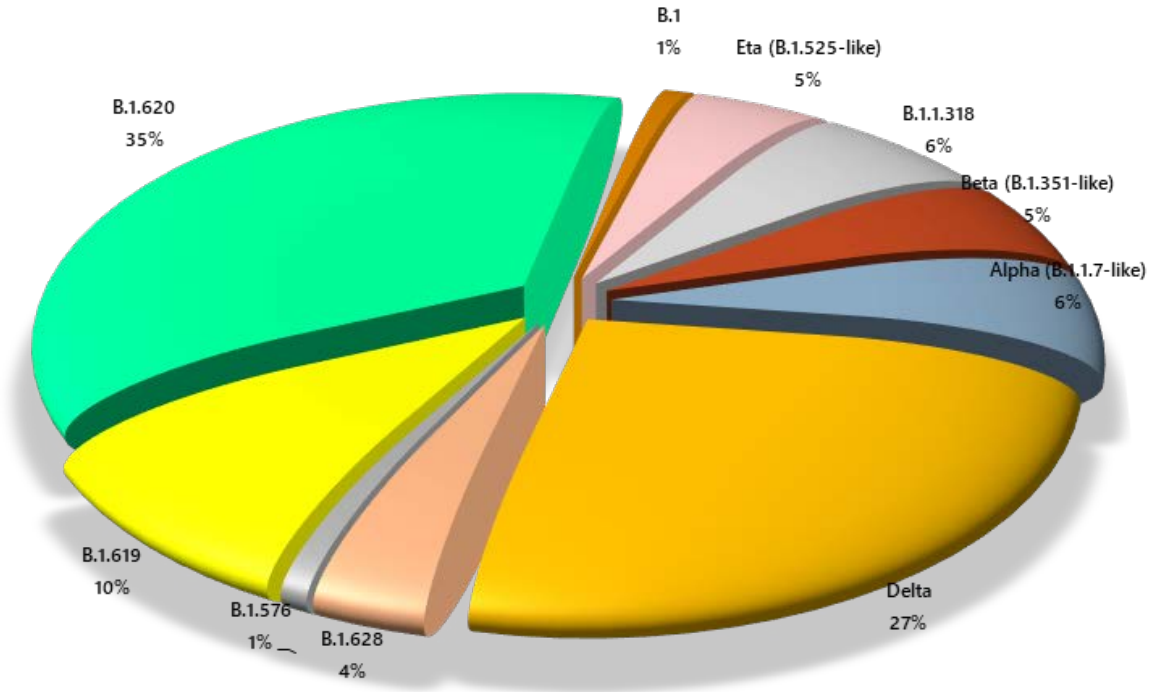
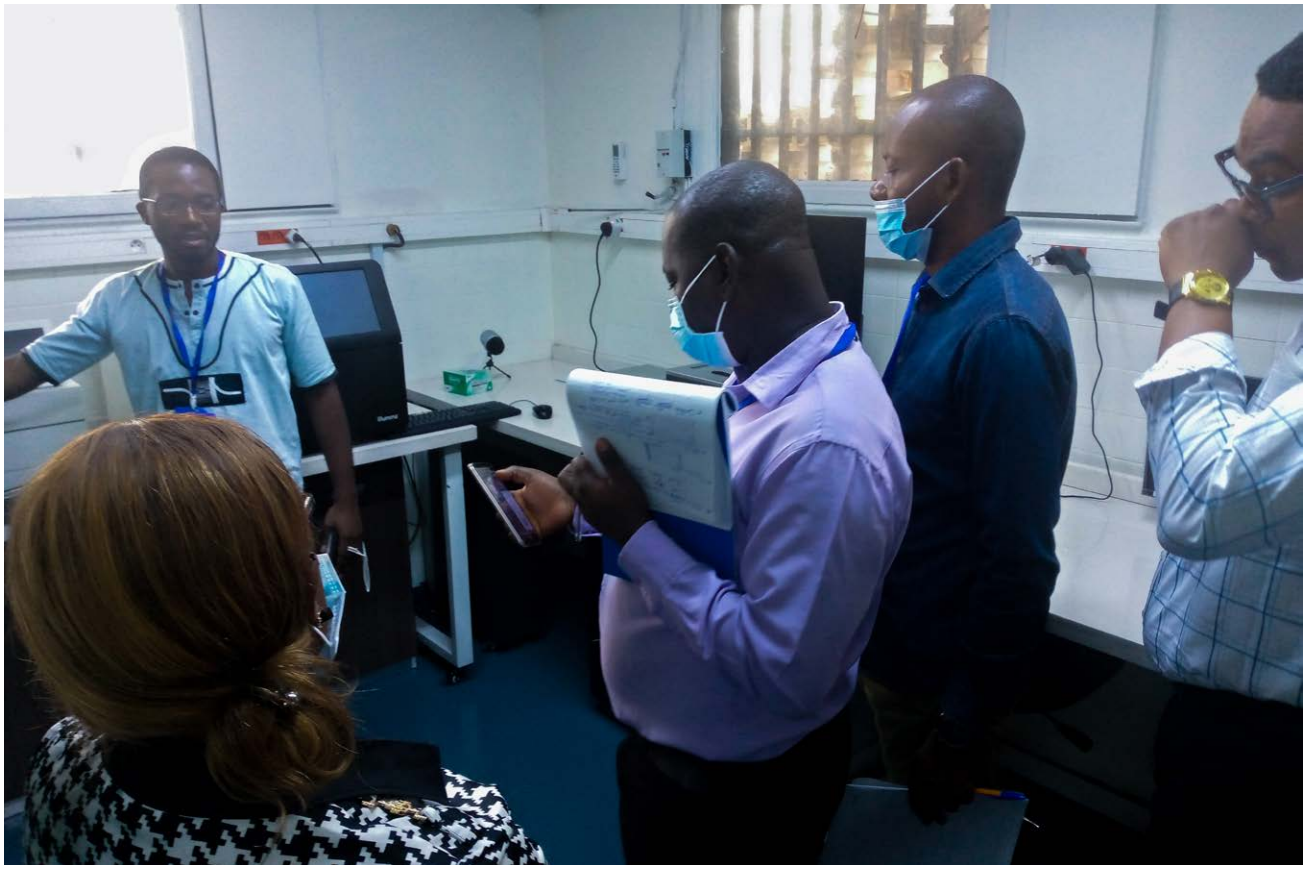


Figure 19 : Distribution of variants detected in the samples of the 2nd expedition Cameroon

iii. Formation

This year, the sequencing laboratory received several guests from Chad and the Republic of Congo to build capacity in SARS-CoV-2 genomic surveillance. Participants were trained in laboratory manipulations according to the Midnight protocol on the Nanopore platform, and introduced to bioinformatics analyses on the artic pipeline.



Country	Number of staff	Training period
Chad	3	From October 13 to 31, 2021
Republic of Congo	2	From October 20 to November 04, 2021
Republic of Congo	1	From 04 to 25 November 2021
Republic of Congo	1	From November 23, 2021 to January 11, 2022
INRB/ Goma	3	From November 21 to 30, 2021

Table 5 : Distribution of countries according to personnel trained in the Midnight Protocol (ONT) and analyses

vi. Submission

Country	Number of Sequences Submitted
Democratic Republic of Congo	1381 (including 354 of 2020)
Cameroon	125
Chad	41
Republic of Congo	241
Central African Republic	42

Table 6 : Number of Sequences Submitted

3. Direct molecular detection of Polio virus with the Nanopore platform

Poliovirus, an infectious agent that mainly affects children under five years of age who are not fully vaccinated, is responsible for a highly contagious disease, poliomyelitis, causing irreversible disabling paralysis, of fecal-oral transmission and has 3 serotypes (PVS1, 2 and 3).

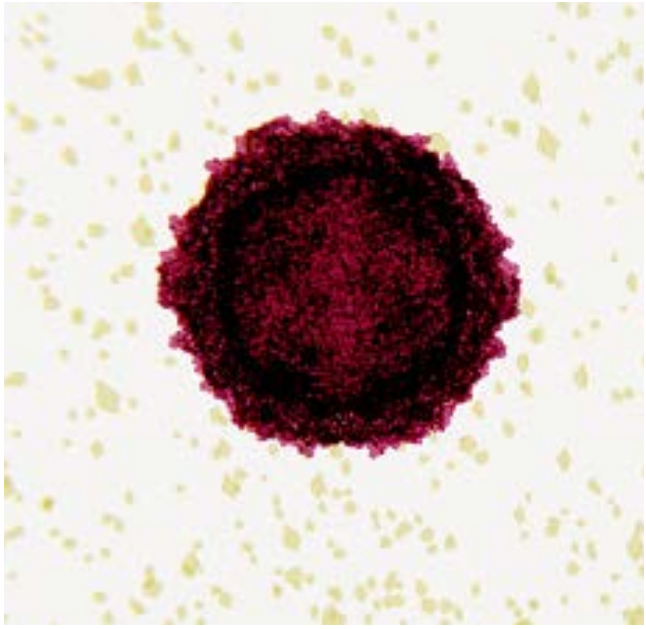
The Democratic Republic of Congo, since the last reported case of Wild Polio Virus in 2011, is also facing several outbreaks of cVDPV marking a quasi-permanent circulation and probably as a result of its low vaccination coverage and late response to outbreaks (Mbaeyi et al., 2019) (Democratique et al., 2015).

Poliovirus surveillance is currently conducted in DRC through culture, with results confirmed by PCR and sequencing conducted externally in South Africa.

In August 2021, a team from INRB's Polio and Infectious Pathogens Gene Sequencing Unit was trained on direct detection of poliovirus from stool samples using the nanopore/MinION sequencing technique (DDNS) as part of the INRB laboratory capacity building project for real-time disease surveillance in partnership with the Bill and Melinda Gate Foundation (BMGF).

This pilot study was preceded by online training and was conducted in 2 phases: retrospective and prospective, and allowed the analysis of more than 2000 stool samples in 4 months. This offered significant advantages in terms of logistics, sensitivity and specificity. Indeed, this experience allowed the reduction of the delay of the result in an average of about 4 days compared to the algorithm of the cell culture used in the surveillance of Acute flaccid paralysis which is done until then only at the INRB on the whole country of 2.345.000 square kilometers. This leads to long intervals between sample collection and detection and response to the epidemic.

The sensitivity and specificity of this Protocol for direct detection of poliovirus from stool samples based on RT-PCR and Embedded PCR of VP1, followed by sequencing of the VP1 region of the Poliovirus with a nanopore/MinION (DDNS), compared to current cell culture based tests and qPCR was also evaluated during a study conducted in Pakistan. The conclusion for this experiment led to a potential method to replace this algorithm and offering a number of significant advantages (Shaw et al., 2020).



Poliovirus Illustration

a. Results of the analyses of this pilot study

The sum of the samples analyzed in 29 Runs for an average duration of 4 Days between the beginning of the laboratory analysis and the return of the results.

Samples analyzed	Workforce
RETROSPECTIVES	283
PROSPECTIVES	2089

Poliovirus Serotype	Sensibility (%)	Number of samples positive by culture
1	100	13
2	100	4
3	83	24

Table 7 : Sensitivity by serotype in the retrospective study

Serotype 1

Spec: 99.8%
Sen: 75%

		DDNS	
		-	+
Culture, ITD and Sanger sequencing	-	2.081	4
	+	1	3

Serotype 2

Spec: 99.9%
Sen: 96%

		DDNS	
		-	+
Culture, ITD and Sanger sequencing	-	2.063	2
	+	1	23

Serotype 3

Spec: 99.7%
Sen: 93%

		DDNS	
		-	+
Culture, ITD and Sanger sequencing	-	2.067	7
	+	1	14

Table 8 : Sensitivity of DDNS compared to culture by serotype

Serotype 3

Spec: 99.7%
Sen: 93%

		DDNS	
		-	+
Culture, ITD and Sanger sequencing	-	2.063	2
	+	1	23

Table 9 : Sensitivity and specificity of DDNS, VDPV2

b. Results of the analyses of this pilot study

- Costs per sample decrease as sequencing runs include more samples;
- More samples decrease the price per sample, stabilizing at about $12-14 per sample;

This price includes reagents, pipette tips, PCR plates, sequencing kits, etc. Prices based on website prices, with no negotiated discounts.

4. Confirmation of the Monkeypox outbreak in Maniema

Monkeypox virus is a member of the genus Orthopoxvirus in the family Poxviridae. Monkeypox is a largely self-limiting disease for which there is no specific treatment. The first human case was recorded in 1970 in the DRC, where it is endemic in the equatorial forest with a case-fatality rate of more than 11%.

The provinces that have reported the highest number of suspected cases are Sankuru, Mai-Ndombe, Equateur, Tshuapa and Mongala.

This year, an outbreak has occurred for the first time in Maniema province, neighboring Sankuru, since November 2021. Samples have been sent to INRB. Monkeypox genome readings were detected in 5 of the 6 blood samples that tested positive for orthopox. However, these samples had relatively low viral loads.

5. Confirmation of the plague outbreak in Ituri province

Plague is a bacterial zoonosis caused by Yersinia pestis, usually found in small mammals and the fleas that parasitize them. There are 2 main clinical forms: bubonic plague and pneumonic plague. The former is the most common and is characterized by a painful swelling of the lymph nodes, the "buboes".

Plague can be very serious in humans, with a case-fatality rate of 30% to 60% for the bubonic form and is almost always fatal in its pulmonary form if left untreated. The 3 main endemic countries are currently Madagascar, the Democratic Republic of Congo and Peru. In the DRC, Ituri province reported 578 cases in 2020 and 2021, resulting in 44 deaths.

During the month of September, INRB received samples of which 23 suspect and available samples were sequenced. The preparation of the libraries was performed using the Illumina RPIP kit with the Illumina Respiratory Panel probes provided with the kit. Fastq files were uploaded to Illumina Basespace Sequence Hub and the Explify IDbyDNA online application for de novo assembly. Of the first 23 samples, 3 were plague PCR positive including 1 with a high bacterial load and in which Yersinia pestis reads were detected.

IV. Challenges and Perspectives

The major challenges remain:
- Supply of reagents and related logistics
- The lack of promptness in the transmission of epidemiological data
- The lack of respect of the cold chain for the samples sent, causing degradation and deterioration of the samples.

In response to these challenges, the laboratory intends to :
- Increase the SARS-CoV-2 sequencing capacity,
- Expand SARS-CoV-2 and polio genomic surveillance activities in the provinces and specifically in North Kivu. This will be preceded by an upgrade of the cold chain and a status report on the implementation of activities;
- The mastery and deployment of Polio detection in other provinces which would be a perfect ideal to further shorten the time to results;
- Sequencing the complete poliovirus genome;
- Extend the sequencing to the detection of enteroviruses and other viruses of the digestive sphere as well as vaccine preventable diseases.

V. Conclusion

The INRB Sequencing Laboratory has been a major asset in the surveillance of pathogens with epidemic potential in the year 2021. Despite the difficulties encountered and the challenges that remain, this laboratory aims to become a Regional Center of Excellence and a world reference in public health research and thus contribute to the well-being of all humanity.

Thanks

The Pathogen Genomics Laboratory would like to thank all its partners for their support to the different activities carried out during this year. We would like to thank the various National Public Health Laboratories (Chad, CAR, Republic of Congo and Cameroon) for their collaboration. We also think of our collaborating departments of the INRB (Virology, Baccteriology) and the Technical Secretariat of the Response against Covid-19 in the Democratic Republic of Congo.

Visit of Professor Jean-Jacques Muyembe, Director General of INRB to the Sequencing laboratory

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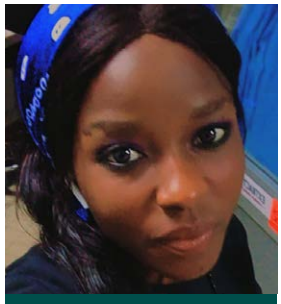

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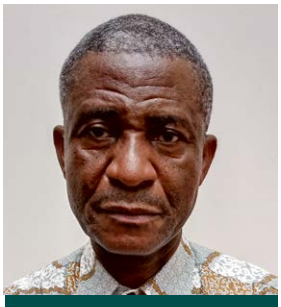

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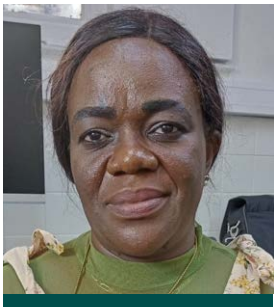

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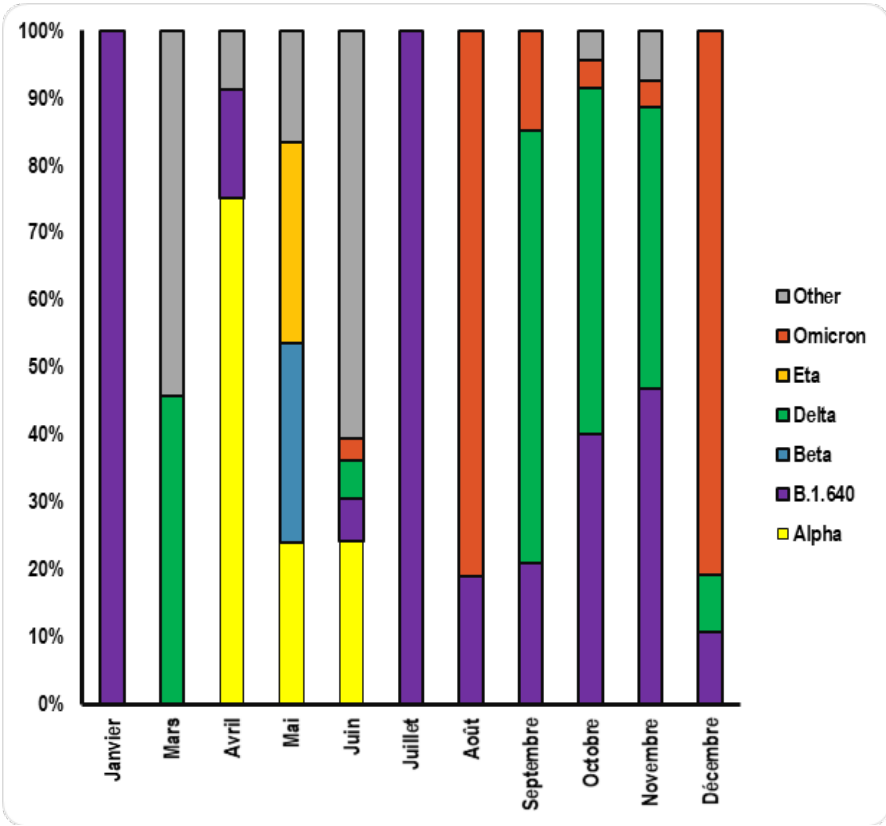
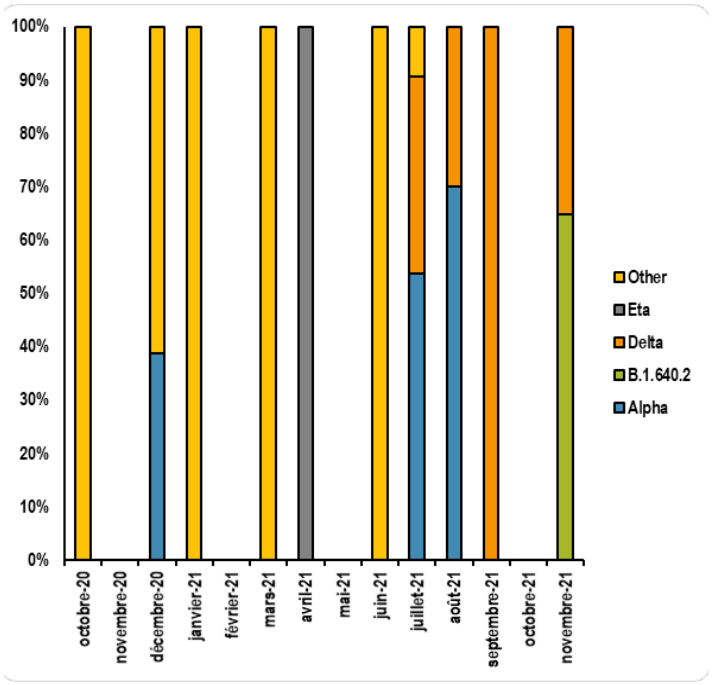
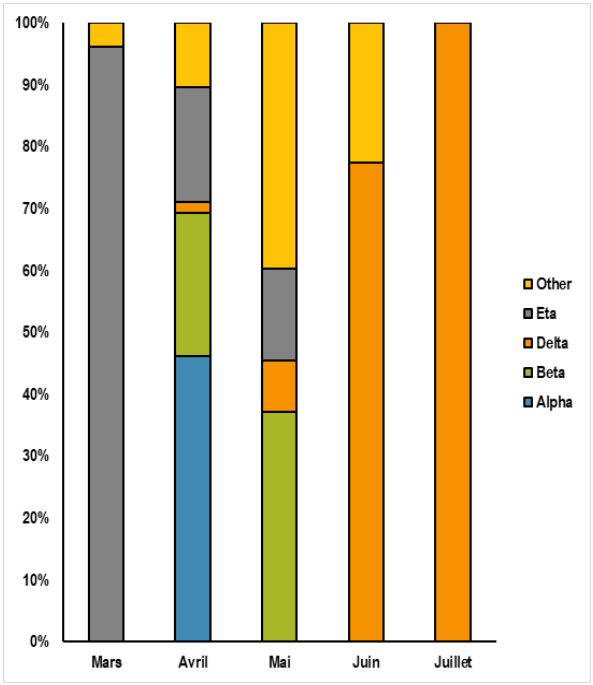
Appendices



Republic of Congo



Chad



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