Research Article

Translational Medicine : Open Access

Virological Diagnosis of SARS-CoV-2 in a Tunisian Orthopedic Institute

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Submitted: 2023, Apr 29; **Accepted:** 2023, May 10; **Published:** 2023, Jun 05

Citation: Kilani, H., Kaoual, S., Nakaa, H., BenLamine, Y., Chatti, L., et al. (2023). Virological Diagnosis of SARS-CoV-2 in a Tunisian Orthopedic Institute. *Transl Med OA,* 1(1), 01-08.

Abstract

Background: *The objective of this study was to investigate the epidemiological profile of the virological diagnosis of COVID 19 at Mohamed Kassab Institute of Orthopedics.*

Methods: *The virological diagnosis was performed by RT-PCR from november 2020 to september 2021 on 33505 nasopharyngeal swabs which different reagent kits were used: Allplex® 2019 nCoV Assay, WANTAI® SARS-CoV-2 RT-PCR, WONDFO® 2019- nCoV Real-Time RT-PCR Assay, GENESIG® Real time PCR coronavirus (COVID-19), GENESIG® COVID-19 2G . The duration from onset to laboratory test in COVID-19 suspected cases and contact individuals ranged from 0 to 14 days with a median of 3 days. The present study provides some genetic information on the lineages of SARS-CoV-2 that circulated in Tunisia over 6 months from april to september 2021. Lineages were assigned for 13 samples using whole-genome sequencing, partial S gene sequencing.*

Results: *A total of 33505 PCR tests were performed from five governorates including Manouba which represented 88.2% (29560). The positive samples were 35% (11695) and 57% (19223) were negative. The sex ratio of confirmed cases was 0.9 (5540/6155). The age group 20-65 years was the most represented with 65.9%. For sequencing her we describe the third wave was marked by the predominance of the Alpha VOC, and the fourth wave was characterized by the predominance of the Delta VOC. This study adds new genomic data to the global context of COVID-19, particularly from the North African region, and highlights the importance of the timely molecular characterization of circulating strains.*

Conclusion: *This study adds new genomic data to the global context of COVID-19, particularly from the North African region, and highlights the importance of the timely molecular characterization of circulating strains.*

Keywords: Diagnostics, Sars-Cov-2, Tunisia, Variant Of Concern, Nasopharayngeal Swabs, Reagent Kits.

1. Introduction

The COVID-19 pandemic first emerged in December 2019 in China, and then rapidly spread to other countries in the world including Tunisia. In the late December 2019, a novel virus called Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), also known as 2019 novel coronavirus (2019-nCoV), was reported with an unidentified source [1]. The genomic sequence of this newly emerged virus is highly similar to that of severe acute respiratory syndrome coronavirus (SARS-CoV) with a 79.6% sequence identity [2] (figure 1). This causes symptoms such as cough and fever, severe pneumonia, and death. The WHO reported that more than 280 million cases of COVID-19, including approximately 5,4 million deaths, have occurred as of december 2021.

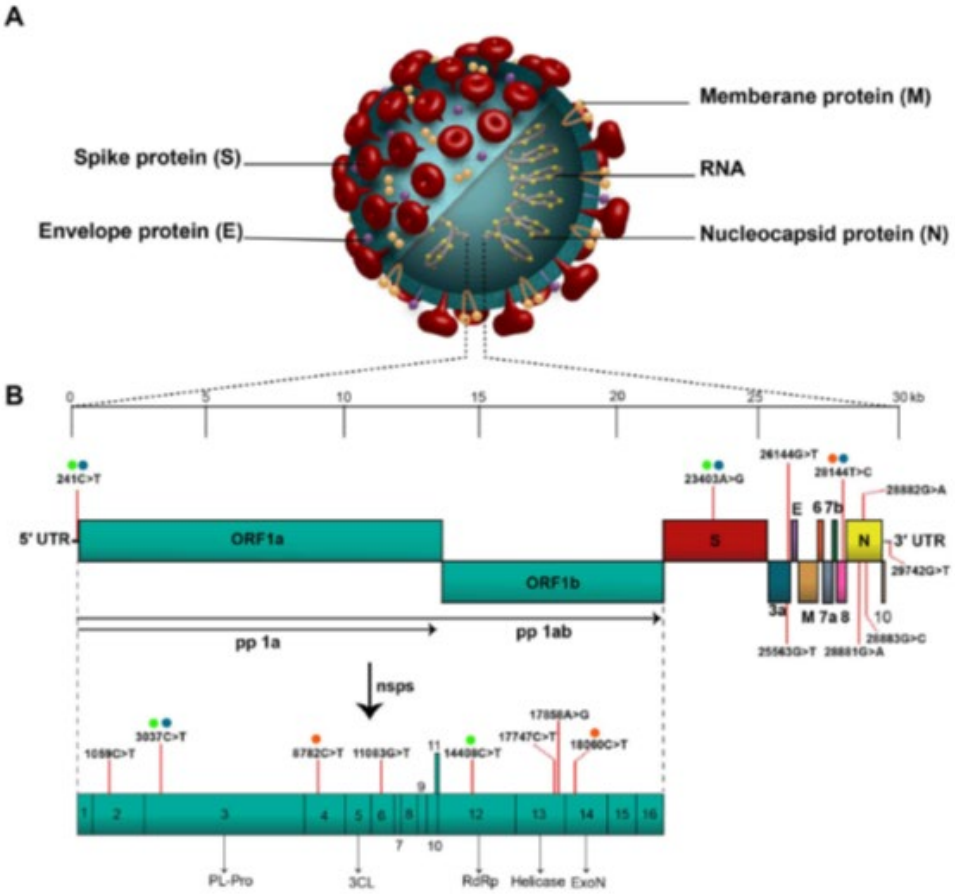

Figure 1: Schematic presentation of structure and genome organization of SARS-CoV-2 based on reference sequence (EPI_ISL_412026) [2].
(A) The virion is covered by the spike (S) proteins as well as the membrane (M) and envelope (E) proteins are placed among the S proteins in the virus envelope. The genomic RNA is surrounded by phosphorylated nucleocapsid (N) proteins inside phospholipid bilayers.
(B) The SARS-CoV-2 genome (29903 nucleotides) comprises of the 5′ UTR, ORF1a/b encoding 16 nsps for replication, four genes that encode structural proteins including S, E, M, and N proteins, six accessory genes that encode six accessory proteins such as ORF3a, ORF6, ORF7a, ORF7b, ORF8, and ORF10, as well as the 3′ UTR. The location of the seventeen high-frequency mutations and co-mutations reported in the literature are shown on the genome by vertical red lines and circles with similar color, respectively.
Abbreviations: SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; 5′ UTR, 5′ untranslated region; OFR, open reading frame; nsp, non-structural protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The first whole-genome sequence of SARS-CoV-2 was published on 5 January 2020, and since then, the analysis of viral sequences worldwide has been continuous, with more than 2.5 million complete genomes currently available in public databases, such as the GISAID platform [2-5]. Molecular analysis has shown significant genetic variability of the SARS-CoV-2 virus due to the accumulation of mutations over time. Most of these changes have little to no impact, but some mutations have an impact on viral properties and lead to an increase in virus transmissibility, more severe infection, a potential reduction in vaccine or immune effectiveness and/or escape from molecular diagnosis.

Variants that have acquired at least one of these characteristics are named variants of concern (VOCs) and require special monitoring. In addition, other variantsare classified as variants of interest (VOIs), variants under monitoring (VUMs) or variants under investigation (VUIs) [6-8]. By July 2021, four major variants of concern (VOCs) had been described that led to increased surveillance efforts worldwide [9]. The Alpha variant, B.1.1.7 lineage (20I/501Y.V1, VOC-202012/01), also known as the UK variant, has an unusually high number of mutations and is more transmissible than the wild-type virus [10]. The Beta variant, B.1.351 lineage (501.V2, 20H/501Y.V2, VOC-202012/02), first detected and reported in South Africa in early October 2020, shares several mutations with B.1.1.7 and reduces vaccine effectiveness to some extent [11,12].

The Gamma variant, P.1 lineage (20J/501Y.V3, VOC-202101/02), emerged in December 2020 in Brazil; it has 10 mutations in the spike protein that may affect its ability to be recognized by antibodies [13-15]. The Delta variant (B.1.617.2, AY.1 and AY.2 VOC-21APR-02) was first described in India and then widely spread all over the world [11,12]. This variant has eight mutations in the spike protein and is characterized by increased transmissibility in comparison with the Alpha variant [11,12]. In November 2021, a new VOC designated the Omicron variant

(B.1.1.529) was first described in Botswana and in South Africa. This variant has rapidly spread all over the world and is presently the most frequently detected worldwide [16,17]. Therefore, the molecular monitoring of circulating strains is crucial for the timely identification of the emergence of novel SARS-CoV-2 variants. After a small decrease in disease incidence in February 2021, the country experienced a third wave of COVID-19 with the introduction of the Alpha variant in March 2021 and then fourth and fifth waves after the introduction of Delta and Omicron in May and December 2021, respectively.

Here, the objective of this work was to investigate the epidemiological profile of the virological diagnosis of COVID 19 at the Mohamed Kassab Institute of Orthopedics. This is a retrospective study from November 2020 to September 2021 and includes suspected COVID-19 cases. Furthermore we provide genetic some information on the lineages of SARS-CoV-2 that circulated in Tunisia during a period of 6 months (April 2020–September 2021) covering three and the four waves.

2. Material and Methods

2.1 Clinical Specimens

This study is based on nasopharyngeal samples tested in the Laboratory of medical Biology at Mohamed Kassab Institute of Orthopedics from November 2020 to September 2021. Nasopharyngeal swab samples were collected from suspected COVID-19 cases in fives governorates in Tunisia; Tunis, Manouba, Bizerte, Sfax and Kbeli.

Samples were mixed in 2 mL of viral transport media (VTM), consisting of Hanks' balanced salt, 0.4% fetal bovine serum, HEPES, antibiotic and antifungal agents. Samples were transported at 2–8 °C to the laboratory for processing within a few hours.

The detection of SARS-CoV-2 by conventional real-time RT-PCR using a different reagent kits: Allplex® 2019 nCoV Assay, WANTAI® SARS-CoV-2 RT-PCR, WONDFO® 2019- nCoV Real-Time RT-PCR Assay, GENESIG® Real time PCR coronavirus (COVID-19), GENESIG® COVID-19 2G [18].

2.2 Viral RNA Extraction

MagLEAD 12gC automated extraction platform (Precision System Science, Chiba, Japan) was used to extract SARS-CoV-2 RNAs from 200 µL of nasopharyngeal and throat swabs. Extraction was performed according to the manufacturer's instructions. Viral RNA was eluted with 100 µL buffer and used for RT-PCR assay [19].

AlphaPrep ™ VIRAL DNA/RNA Extraction Kit (Model: VDR-B096V) was used for extraction of nucleic acids from various cell types. The extracted nucleic acids are applicable PCR, real-time PCR, and enzymatic reaction etc.

2.3 Preparation of Plate

Remove the sealing cover and add 200 µl of sample and 20 µl of proteinase K to the 1st or 7th well in the plate. Insert the plate correctly in the instrument (GenMagBio RNA extractor reagent kit with reliable quality)

2.4 Extraction of Nucleic Acids

After inserting the plate, close the door of the instrument and enter the correct program. When operation is finished, remove the plate from the instrument and then transfer 80 µl of extracted nucleic acids 6Th or 12th to the 1.5 ml tube.

SARSCoV2 RNA detection using real time RTPCR and Variant Detection by Partial Sequencing of the S Gene The SARS-COV-2 RNA sequences are amplified by the presence of specific primers. These were designed to amplify in vitro a specific region of nucleic acid: the structural genes. Depending on the type of used kit [20].

RT-PCR is the gold standard in the diagnosis of COVID-19. However, this test does not detect SARS-CoV 2 mutations, hence the genomic sequencing was maked by the laboratory of Clinical Virology, WHO Reference Laboratory for Poliomyelitis and Measles in the Eastern Mediterranean Region, Institut Pasteur de Tunis.

Amplification by standard PCR and partial sequencing using Sanger technology was used for 13 samples collected from April to september 2021, as described previously [21]. The 648-nucleotide-long S gene sequence encodes for the 477 to 693 amino acid residue region of the S protein. It includes key positions and allows the detection of the most important mutations characterizing most VOCs, VOIs and VUMs.

3. Results

A total of 33505 PCR tests were performed, from five governorates including Manouba which represented 88.2% (29560) (Figure 2).

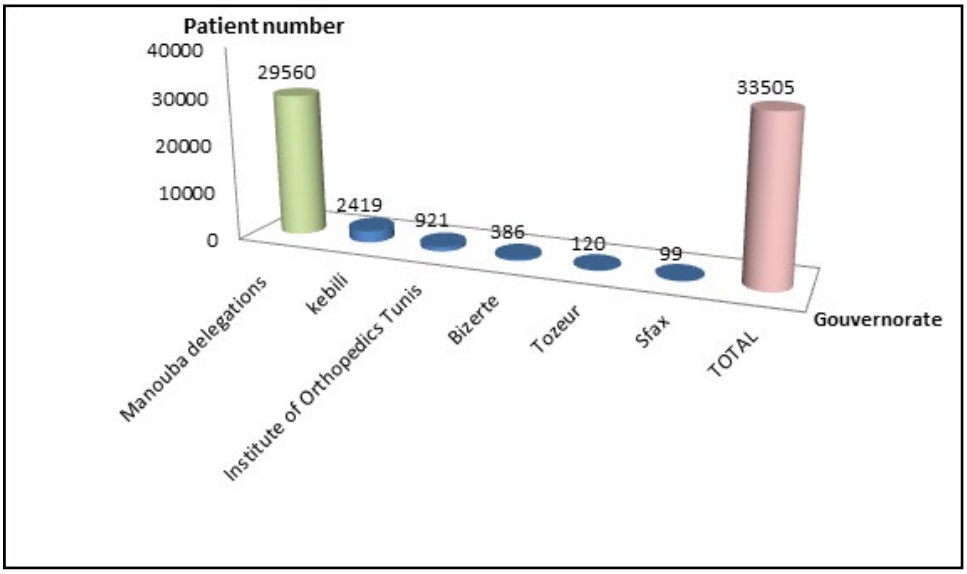

Figure 2: Breakdown of withdrawals by governorate.

The positive samples were 35% (11695) and 57% (19223) were negative.
The sex ratio of confirmed cases was 0.9 (5540/6155).
The age group 20-65 years was the most represented with 65.9% (Table 1).

Age Range	Number	%
7-19 ans	625	5,34
20-55	6804	58,18
55-65	902	7,72
65-89	617	5,27
not mentioned	2747	23,48
Total	11695	100

Table 1: Distribution of confirmed cases of COVID-19 by age.

A first peak between January and February 2021 showed a positivity rate of 22.4% and a second peak between June and July with 41.2% positivity (Figure 3).

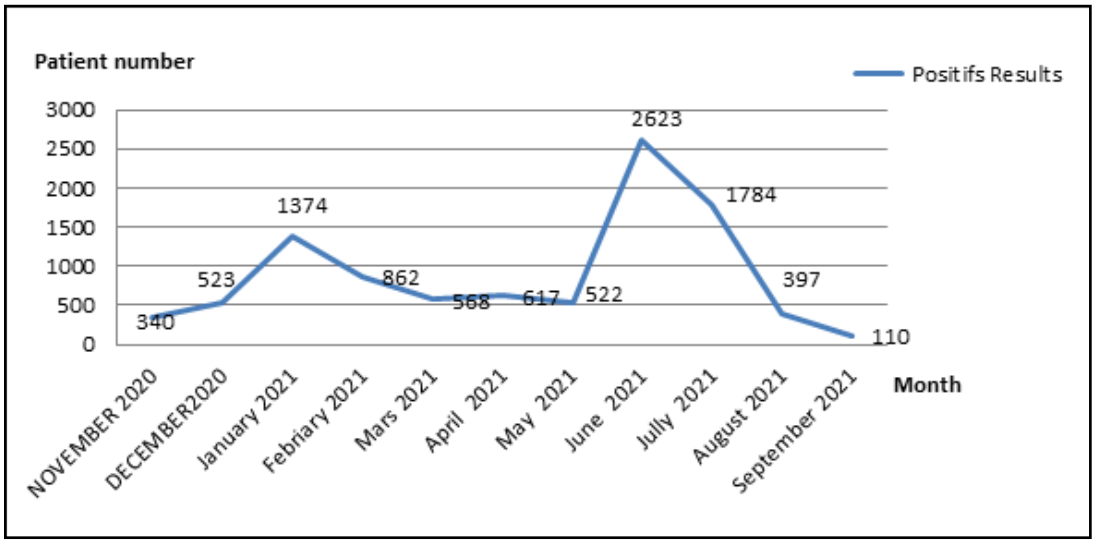

Figure 3: Evolutionary curve of confirmed COVID-19 cases.

Nine out of ten of the COVID-19 positive samples sent from April were British B.1.1.7 (UK) variants of concern (Alpha) which have an accumulation of four substitution mutations at the nitrogen base levels 501-570-614- 681 and a variant A.27 (VOI) which presents four mutations by substitution at the levels of the nitrogenous bases 507-653-655 compared to the S gene of the Wuhan parent strain.\

The other three positive samples from September were B.1.617.2-like of concern (Delta) which have an accumulation of three substitution mutations; The effect of mutations in SARS-CoV-2 genomes highlighted similar profiles with D614G spike (S) variants which as the most change (Table 2).

Month	Sample number	Sequences	Pango Lineage	Mutations
April	1	S130	B.1.1.7 (UK)	N501Y-A570D- D614G-P681H
	2	S132	B.1.1.7 (UK)	N501Y-A570D- D614G-P681H
	3	S147	B.1.1.7 (UK)	N501Y-A570D- D614G-P681H
	4	S151	B.1.1.7 (UK)	N501Y-A570D- D614G-P681H
	5	S152	B.1.1.7 (UK)	N501Y-A570D- D614G-P681H
	6	S154	B.1.1.7 (UK)	N501Y-A570D- D614G-P681H
	7	S156	B.1.1.7 (UK)	N501Y-A570D- D614G-P681H
	8	S161	B.1.1.7 (UK)	N501Y-A570D- D614G-P681H
	9	S660	B.1.1.7 (UK)	N501Y-A570D- D614G-P681H
	10	S159	A.27 (VOI)	N501Y-A653V-H655Y
September	11	S-1516	Delta (B.1.617.2-like)	L452R-T478K-D614G
	12	S-1519	Delta (B.1.617.2-like)	L452R-T478K-D614G
	13	S-1524	Delta (B.1.617.2-like)	L452R-T478K-D614G

Table 2: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) lineage distribution in Tunisia in april and September months 2021.

This is confirming with the emergence of variants of concern (VOCs) and variants of interest (VOIs) in Tunisia between March 2020 and July 2021 (figure 4). In the background, is the number of positive cases detected in Tunisia according to the statistics published in the World Health Organization (WHO) website [22].

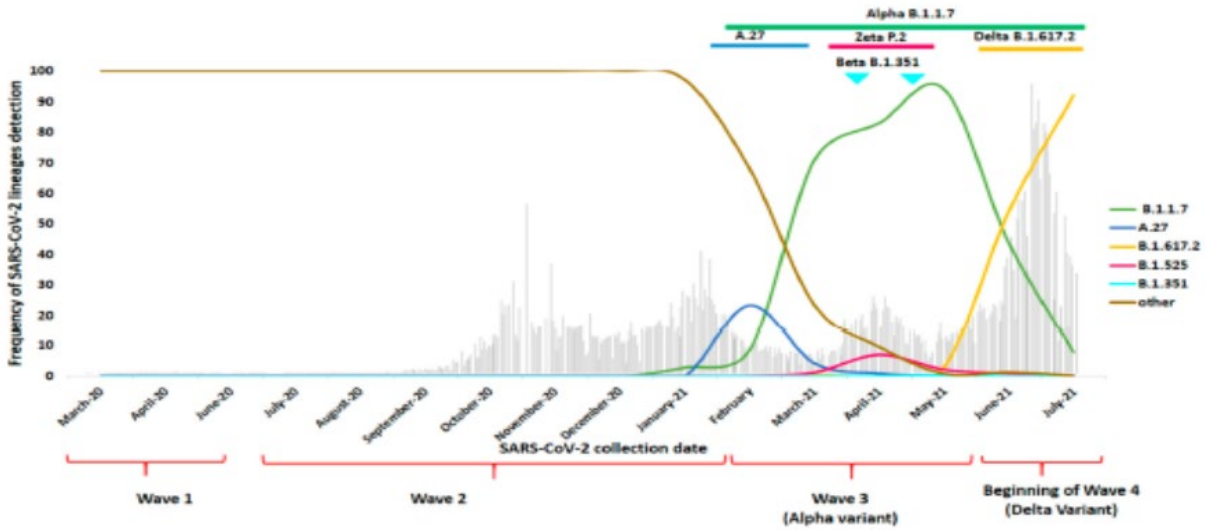

Figure 4: Emergence of variants of concern (VOCs) and variants of interest (VOIs) in Tunisia between March 2020 and July 2021. In the background, is the number of positive cases detected in Tunisia according to the statistics published in the World Health Organization (WHO) website [22].

4. Discussion

Molecular tests are the standard laboratory diagnosis to confirm SARS-CoV-2 infection; RT-PCR assays for SARS-CoV-2 RNA detection in clinical specimens are widely used in COVID-19 diagnostic laboratories. In this retrospective observational study, we describe the SARS-CoV-2 that circulated in fives governorates in Tunisia for 11 months after its first introduction to the country in March 2020. First, Tunisia, a small country with an area of 163,610 km2 and a population of approximately 12 million, has a strategic geographic location, which makes it a junction point between the Arab world, Africa and Europe. Furthermore, it is known for its history of economic and cultural transactions, particularly with European and neighboring countries. In addition, Tunisia was experiencing an economic and political crisis that prevented total lockdown for long periods, and the introduction of the anti-SARS-CoV-2 vaccine to the population was relatively late.

The succession of the different waves observed in Tunisia is similar to the global picture of COVID-19 infection (https://covid19.who.int/ (accessed on 30 August 2021)). In addition, the same picture of the circulation of several viral lineages has been reported in several countries around the world, such as the Czech Republic, Cyprus, the UK, Russia, South Africa and countries from the Middle East and North Africa (MENA) region [23-28].

The exchange between countries has played a crucial role in the importation of new lineages and their rapid spread across countries. This phenomenon is characteristic of airborne viruses. Countries that have succeeded in stopping their transmission are those that have applied drastic measures, such as China and South Korea, or other countries that were able to contain the virus during the first phases of the pandemic with full containment, border closures and the minimization of any contact, even between non-infected people [23-28].

In our study we observed the epidemic had first peak between January and February 2021 and a second peak between June and July with 2627 and 4825 confirmed cases respectively, which is close to the epidemiological situation in Tunisia. Such as we showed a positivity rate of 35% and more than half of the confirmed cases were in the age group of 20-65 years. Studies done in Wuhan showed that the majority of Covid-19 patients are adults; their average age was 55.5 years. Elderly subjects accounted for only 10.1% of patients. Children are less frequently affected and less severely, but some authors have described the involvement of infants under one month of age [29].

The sex ratio was 0.9. Different results were found by Ketfi A. et al in Algeria with a sex ratio of 1.4 and by Nikpouraghdam M. et al in Iran with a sex ratio of 1.9 [30-31]. RT-PCR is the gold standard in the diagnosis of COVID-19. However, this test does not detect SARS-CoV 2 mutations, hence the genomic sequencing was maked by the laboratory of Clinical Virology, WHO Reference Laboratory for Poliomyelitis and Measles in the Eastern Mediterranean Region, Institut Pasteur de Tunis.

In our study we researched the partial S gene sequencing for 13 specimens with Ct values less than 30 that were collected throughout April and September 2021. Nine out of ten of the COVID-19 positive samples sent from April were British B.1.1.7 (UK) variants of concern (Alpha) which have an accumulation of four substitution mutations at the nitrogen base levels 501-570-614- 681 and a variant A.27 (VOI) which presents four mutations by substitution at the levels of the nitrogenous bases 507-653-655 compared to the S gene of the Wuhan parent strain.The other three positive samples from September were B.1.617.2-like of concern (Delta) which have an accumulation of three substitution mutations; The effect of mutations in SARCoV-2 genomes highlighted similar profiles with D614G spike (S) variants which as the most change. Moreover, other lineages circulated for long periods, such as B.1.160 and B.1.177, which took an important place in the lineage landscape circulating in Tunisia. These lineages circulated from September 2020 until mid-2021 without any impact on the overall epidemiological situation. B.1.160, known as 20A/EU2, is one of the main variants first reported in Europe [32,33]. The B.1.177 lineage, mostly detected in Europe, was first detected in early 2020 and is currently classified into more than 80 sub-lineages [32]. Further molecular characterization of a higher number of viruses will be of great interest to better characterize these two lineages.

In May 2021, the Delta variant, characterized by L452R and P681H amino acid substitutions in the spike protein, was detected in the country and rapidly displaced the Alpha variant, becoming the dominant variant in June–July 2021. This VOC, first detected in India in early 2021, became the most frequently detected variant in many countries [5,34,35]. Indeed, it was demonstrated that the Delta variant emerged faster than the Alpha variant and dominated the variant landscape worldwide. In the present study, the emergence of the Delta variant defined the fourth wave of SARS-CoV-2 infection in the country and participated in the resurgence of SARS-CoV-2 cases.

The circulation of the Delta variant coincided with high transmissibility and with a large number of severe disease cases. In fact, infection with the Delta variant is characterized by the generation of an average of 6 times more viral RNA copies per milliliter than Alpha infections [12]. The Alpha VOC, first detected in the United Kingdom in late 2020, is defined by an N501Y amino acid substitution in the spike protein that increases its transmissibility. The Alpha VOC had become the dominant global variant by early 2021 [5,28,30,31]. In Tunisia, the detection of the Delta variant decreased from August to December 2021 (data not shown). The disease incidence increased again with the introduction of Omicron, which was first detected in early December and caused a new wave with a much higher transmission rate.

In order to limit the progression of the SARS-COV-2 virus in the population, it is essential to reinforce the application of the "Test-Trace-Isolate" strategy: Ø each person with symptoms suggestive of COVID-19 or having the slightest doubt must carry out a virus test as soon as possible. ØThe times for screening and isolating cases and their contact must be reduced for a greater effectiveness of this strategy. ØPending the results, people must isolate themselves and contact must be reduced to a strict minimum.ØThis behavior must be coupled with adherence to all

barrier gestures, in particular the wearing of mask, participation in contact identification measures, compliance with isolation measures for infected persons, persons likely to be infected or contacts classified at risk.

Preventive measures must be applied to minimize public health risks such as; Continue braking strategy, Respect health protocols, Health monitoring should be associated with the variousbarrier and physical distancing measures and Base prince: balance between the probability of occurrence of cases and application of general measures. This study describes the Tunisian experience in the molecular surveillance of SARSCoV2. The generated genomic data contribute to the enrichment of the globally published data on SARS-CoV-2 circulation, particularly in North Africa.

5. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
Les auteurs déclarent qu'ils n'ont pas d'intérêts financiers concurrents connus ou de relations personnelles qui auraient pu sembler influencer les travaux rapportés dans le présent document.

Funding: This study was funded by the authors.

Author Contributions: Original draft preparation writing, editing Hajer Kilani. Review Salma.Kaoual methodology Hajer. Kilani, Hadhemi Nakaa, validation visualization supervision Sophia BouhalilaBesbes All authors read and approved the final version of the manuscript for submission.

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