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Original article

Genomic Analysis of SARS-CoV-2 in Libya: Spatiotemporal Clade Dynamics and Mutations Influencing Viral Virulence

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Abstract
The ongoing evolution of SARS-CoV-2 has resulted in emerging variants with increased transmissibility and immune escape. In order to strengthen studies on pandemic evolution and set the public health responses, the incorporation of genomic data from global databases such as NCBI and bioinformatics platforms, e.g., Nextclade, has played an important role. This retrospective study was conducted to investigate the genomic diversity among SARS-CoV-2 sequences in Libya from March 2020 to December 2022, through assigning variant distribution and key mutations with the possible functional implications. Eighty-one (81) high-quality SARS-CoV-2 genomes from Libya were retrieved from NCBI, of which thirty (30) sequences were then analyzed for clade assignment, quality assessment, and mutational profiles. SARS-CoV-2 sequences were classified into lineages by using Pangolin software (V4.x.). All 30 sequences had high quality with minimal sequencing errors. Clade distribution results showed that the dominant variants were 21D (Eta) and 22B (Omicron BA.5) at 50% and 20%, respectively. Other detected sublinages were 21L (16.7%), 22C (10%), 20B (B.1.1, 3.3%), 19B (3.3%). The major nucleotide mutations in clade 21D included C241T (5' UTR; 100%) and C884T (ORF1ab; 93%), and in 22B T670G (ORF1ab; 100%) and C1627T (ORF1ab; 83%). Moreover, the amino acid substitutions included S: E484K (100%) in 21D, S: F486V (100%) in 22B, ORF1ab: P314L (100%) in 21L, and S: D614G (100%) in 22C. Our findings identified a complex genetic diversity in SARS-CoV-2 variants in Libya, which carried important mutational changes, and underscored the increased need for continuous and large-scale sequencing analysis to understand the local and international trajectory of the pandemic.
Keywords. SARS-CoV-2, Libya, NCBI, Viral Variants, Mutation Analysis, Nextclade.

Introduction

The emergence of SARS-CoV-2 in late 2019 began an unprecedented global health crisis, culminating in the World Health Organization declaring COVID-19 a pandemic in March 2020 [1]. Libya had reported its first confirmed case in March 2020, and by early 2023, over 500,000 confirmed cases and nearly 6,400 deaths had been reported across Libya [2,3]. This demonstrates the considerable strain that Covid-19 placed upon Libya's healthcare system across the country and highlight a critical need for effective genomic surveillance systems.

While genomic surveillance is important, Libya is among the least characterized countries in North Africa in terms of SARS-CoV-2 evolutionary analysis, with limited shared genomic data compared to other neighboring countries [4]. Certainly, this lack of Libyan information influences public health decisions and pandemic preparedness. However, SARS-CoV-2 has high evolutionary potential with a potential rate of 1.1×10^{-3} substitutions per site per year [5]. This adaptive capacity has led to the emergence of several subsequent variants with improved transmissibility and immune evasion capacity. Globally, the dominant clades that contributed to the pandemic between 2020 and 2022 were Alpha, Delta, and Omicron; however, genomic surveillance in North Africa revealed a different picture for Libya, which harbored the Eta variant (B.1.525) predominance, and at minor percentages, Alpha, Delta, and Omicron variant strains occurred [4]. Coronaviruses, including SARS-CoV-2, are enveloped RNA viruses that have several distinguishing features. They have extremely large genomes and a complicated lifecycle. In particular, their genome encodes a proofreading system (Exoribonuclease, ExoN), distinguishing them from other RNA viruses, which minimizes transcriptional errors (proofreading) and confers greater genome stability [6]. The viral genome has structural proteins, including the spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, as well as non-structural proteins (nsps) that perform essential functions for virulence, replication, and host interaction [7].

Despite proofreading, SARS-CoV-2 has accumulated a variety of mutations that increase its virulence potential. For example, mutations in the spike protein, including the D614G, favor increased transmission and infection [8, 9]. Other mutations in the spike protein, including E484K, are recurrent indicators of immune escape, by allowing avoidance of neutralizing antibodies, while maintaining strong receptor-binding affinity [10, 11]. Significant mutations are also found in its non-structural proteins; one example is the P314L (or P323L) mutation in RNA-dependent RNA polymerase (RdRp), which could also exacerbate

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replication fidelity [12]. In addition, the mutation at the furin cleavage site is also being examined for its role in virus pathogenicity and effects on clinical disease [13].

Public databases, such as the National Center for Biotechnology Information (NCBI), have become an important resource to find SARS-CoV-2 sequences worldwide [14]. These databases allow researchers to collect viral genomes from different regions across the world and provide the basis for comparative genomic studies. In parallel, bioinformatics tools such as NextClade and Pangolin software allow for identifying and characterizing the viral lineages, mutations, and comparing local isolates against reference genes [15]. Integrating global viral database and bioinformatics tools ensures that even in settings with limited sequencing capacity, valuable insights and dynamics are possible to detect significant amino acid changes and monitor evolutionary trends.

This retrospective study aims to address the gap in genomic surveillance in Libya by providing a descriptive genomic analysis of retrieved Libyan sequences from March 2020 to December 2022, identifying the most prevalent viral clades, and determining the frequency of biologically significant mutations over the study period. This study also aimed to demonstrate how computational assessment of available public data could help to overcome logistical challenges in settings with fewer resources. This research reinforces the regional data to better understand the dynamics of SARS-CoV-2 in the Libyan context and assist in providing well-placed targeted strategies in public health to respond to and/or prevent pandemics.

Methods

Data acquisition

Eighty-one (81) complete Libyan SARS-CoV-2 genomes from March 2020 and December 2022 were retrieved from NCBI Virus Database (https://www.ncbi.nlm.nih.gov/labs/virus/) [data were retrospectively acquired during March 2024]. Filters were performed to include only human-host sequences with a genome length >29,000 bp. Any associated metadata (including GenBank accession, date of collection, and sampling location) was downloaded as CSV for downstream analysis. For example, a metadata entry would be as follows: GenBank ID: ON123456; Virus name: hCoV-19/Libya/Tripoli-01/2021; Collection Date: 2021-05-20; Location: Libya/Tripoli. The workflow of data acquisition, quality control, and genomic analyses is outlined in (Figure 1).

Quality control and filtering

Sequences were screened for completeness and ambiguity. Sequences with >5% ambiguous bases (Ns) or 10% missing data, internal stop codons, or frameshifts were flagged. Nextclade (v3.17.0) quality metrics were also used to mark low-quality sequences. Next, flagged sequences were excluded. After quality control (QC), thirty (30) high-quality sequences including the reference sequence were retained and only 30 sequences comprised the final dataset for downstream analyses.

Analysis of sequences and clade/lineage classification

High-quality FASTA sequences were further examined using Nextclade (v3.17.0); https://clades.nextstrain.org. Each sequence was compared to the Wuhan-Hu-1 reference genome (GenBank accession: MN908947.3 / RefSeq: (NC_045512.2) to determine clade and mutation information, and quality control metrics. The output files from Nextclade (nextclade.tsv and mutations.csv) were utilized to create mutation profiles for each sequence, along with identifying key mutations that define the clades. In addition, the Pangolin tool (V4.x) performed lineage classifications, and any sequences that were not assigned by Pangolin were flagged as unassigned.

Data integration and summary statistics

Nextclade and Pangoline outputs were integrated with NCBI metadata using GenBank accession numbers. We then summarized clade/lineage counts and relative frequencies, tabulated recurrent mutations (when >70% of sequences belonged to the same clade), and calculated QC metrics (mean sequence length, % Ns, etc.). All result tables were generated in Microsoft Excel for use in the manuscript.

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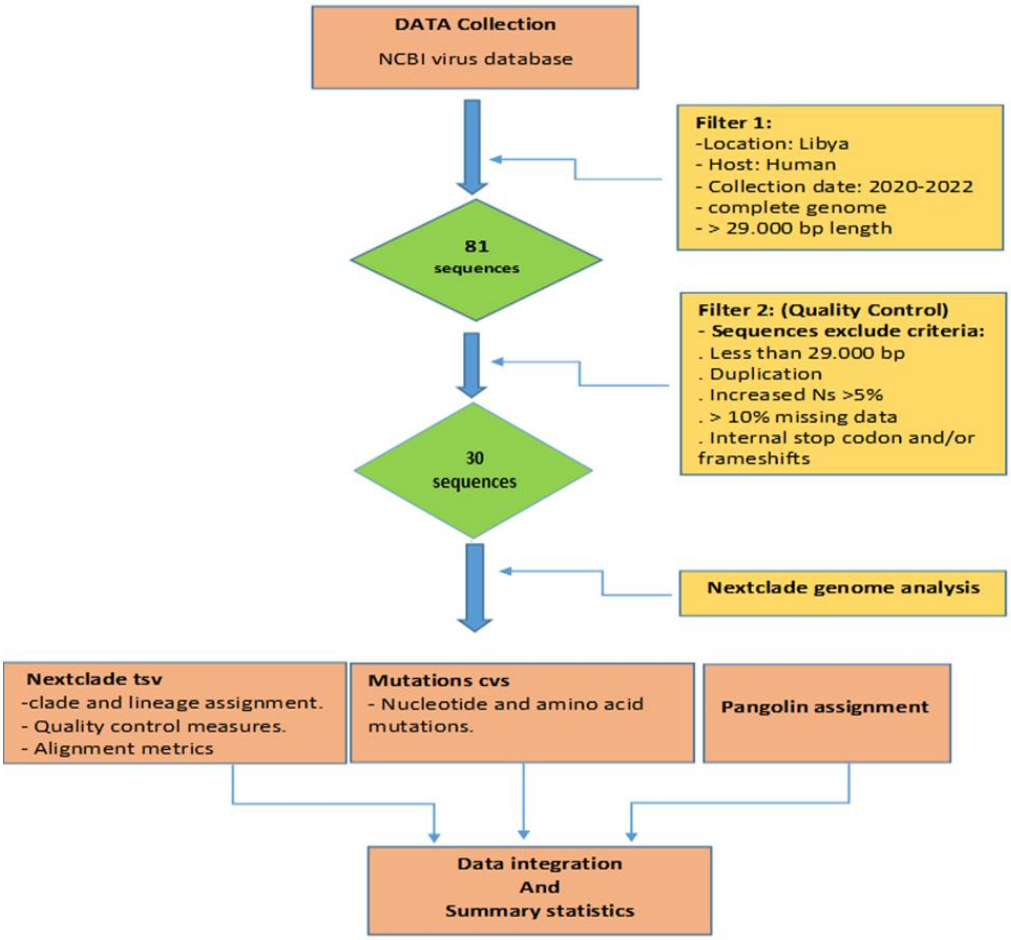

Figure 1. Data source and workflow of sequence analysis. All sequences were retrieved from NCBI. Then filtered and analyzed by Nextclade and used for assigning the clade and the identification of the mutation

Results

Clade distribution and circulation of variants

A retrospective whole-genome sequencing of 30 high-quality SARS-CoV-2 isolates from Libya during the period of March 2020- December 2022 was conducted, and the results of clade distribution indicated the circulation of multiple variants (Table 1). The predominant clade was 21D pangolineage (B.1.525), which represented half the sequenced samples (50%, n=15), followed by Omicron 22B (BA.5.2), which made up 20% (n=5). Other less prevalent clades, such as 21L (16.7%, n=5) and 22C (10.0%, n=3), were detected**.** The early variants 20B (B.1.1) and 19B were also reported but in lower frequencies (3.3%, n=1 each). However, Pangolin failed to assign some clades that were successfully classified by Nextclade.

Table 1. SARS-CoV-2 clade distribution among Libyan isolates

Clade	Pangolineage Classification	Number of Sequences (n)	Percentage (%)
21D (Eta)	B.1.525	15	50%
22B (Omicron)	BA.5.2	5	20%
21L	Unassigned	5	16.7%
22C	Unassigned	3	10%
20B	B.1.1	1	3.3%
19B	Unassigned	1	3.3%

Nucleotide Substitution Patterns

Mutational analysis of Libyan isolates demonstrated multiple clade-defining nucleotide mutations (Table 2). The most common nucleotide mutation detected in clade 21D (Eta) was C241T (5′ UTR) with 100% prevalence. The second most detected nucleotide mutational change (14/15 sequences), with a 93% prevalence rate, was C884T (ORF1ab) in Eta isolates. Moreover, both T670G (ORF1ab; 100%, 6/6) and

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C1627T (ORF1ab; 83%, 5/6) were highly observed in clade 22B. However, a C3737T (ORF1ab; 100%, 1/1) was a unique substitution detected in B20.

Table 2. Representative nucleotide mutation among Libyan SARS-CoV-2 isolates

Clade	Nucleotide Mutation	Genomic Position	Number of Sequences with Mutation	Prevalence (%)
21D (Eta)	C241T	5′ UTR	15/15	100
21D (Eta)	C884T	ORF1ab	14/15	93
22B (Omicron)	T670G	ORF1ab	6/6	100
22B (Omicron)	C1627T	ORF1ab	5/6	83
20B (B.1.1)	C3737T	ORF1ab	1/1	100

Amino Acid Mutations

The amino acid substitution analysis showed a number of substitutional amino acid mutations of potential functional significance (Table 3). All clades of 21D (Eta) possessed the spike protein mutation S: E484K (100%, 15/15). Regarding clade 22B (Omicron BA.5), the ubiquitously detected spike mutation was S: F486V (100%, 6/6). The ORF1ab: P314L mutation was reported in all isolates of clade 21L (100%, 5/5). Lastly, all sequences of clade 22C showed the now ubiquitous S: D614G spike mutation (100%, 3/3).

Table 3. A common amino acid mutation in SARS-CoV-2 sequences from Libyan isolates.

Clade	Amino Acid mutation	Protein	Prevalence (n/N)	Prevalence (%)
21D (Eta)	S:E484K	Spike	15/15	100
22B (Omicron)	S: F486V	Spike	6/6	100
21L	ORF1ab:P314L	ORF1ab	5/5	100
22C	S: D614G	Spike	3/3	100

Quality control (QC) Assessment of Sequencing

All sequences that were assessed in this study met the Nextclade quality control requirements, providing confidence in the accuracy of variant assignments. As indicated in (Table 4), mean QC scores were high across all clades, between 4.32 and 4.78. Missing data was consistently minimal throughout the dataset, consistently below 1% across all samples. None of the coding sequences contained frameshift mutations or premature stop codons, and thus confirmed that the genomic data were sound.

Table 4. Summary of sequencing quality metrics by clade

Clade	*Number of Sequences*	*Mean QC Score*	*Mean Missing Data (%)*	*Frameshift Mutations*	*Premature Stop Codons*
21D (Eta)	15	4.34	0.99	0	0
22B (Omicron)	6	4.78	0.96	0	0
21L	5	4.34	0.99	0	0
22C	3	4.34	0.99	0	0
20B (B.1.1)	1	4.35	1.00	0	0
19B	1	4.32	0.98	0	0

Founder Mutation Analysis

The analysis of founder mutations provided additional confirmation of clade assignments and underlined the genetic signature of the circulating isolates (Table 5). Several nucleotide mutations, including C241T, C884T, and C1498T, and a characteristic set of amino acid mutations such as ORF1ab: P314L and S: E484K, were carried by Clade 21D (Eta). However, clade 22B (Omicron BA.5) exhibited a definitive founder change in T670G and C1627T, as well as amino acid mutations in S: F486V and ORF1ab: T1001I.

Table 5. Founder mutations defining the major SARS-CoV-2 clades circulating in Libya

Clade	*Defining Nucleotide Mutations*	*Defining Amino Acid Mutations*
21D (Eta)	C241T, C884T, C1498T	ORF1ab:P314L, S:E484K
22B (Omicron)	T670G, C1627T	S:F486V, ORF1ab:T1001I

Discussion

The present retrospective study provides a significant contribution to the genomic epidemiology of SARS-CoV-2 in Libya, underscoring both similarities and differences from global and regional trends. In general,

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the period between 2020 and 2022 showed consecutive waves of SARS-CoV-2 Variants of Interest (VOIs): Alpha was prominent in early 2021, succeeded by the highly transmissible Delta, and then the immune-evasive Omicron variant, all of which transformed the pandemic trajectory at a global level [16]. Our genomic analysis showed a distinct trend for SARS-CoV-2 variants circulating in Libya when compared with the viral situation in other North African countries. Early waves in Egypt, Tunisia, Algeria, and Morocco were dominated by Delta (clade GK) and later Omicron (clade GRA), while waves in Libya were characterized by dominance of the Eta variant (B.1.525, clade G). Approximately 50% of the Libyan sequences submitted to GISAID had been classified as Eta, while Alpha, Delta, and Omicron comprised only minor variants [4]. This is also supported by the results of a Libyan study conducted in 2021, which reported 55% of sequenced samples were classified as Eta (B.1.525) and only ~3% classified as Alpha (B.1.1.7) [17]. Principally, Eta was first identified in Nigeria in December 2020. The predominance of the Eta variant in Libya could be a result of introduction via regional travel or trade, as well as the delayed arrival of major waves driven by the other variants of concern in nearby countries[18]. Successively, Omicron sublineages became established in Libya, following worldwide and regional trends [19].

This study identified a variety of Omicron sublineages, including 22B (BA.5.2, 20%), 21L (BA.2, 16.7%), and 22C (10%). Similar variant progression has been reported in Egypt, where BA.1 and BA.2 began to increase, with BA.2 ultimately prevailing [20]. The ultimate displacement of Eta and Alpha by Omicron sublineage underscores the competitive advantages among these variants driven by their spike mutations and adaptive virulence [21]. Particularly, the BA.5.2 variant has been described to have increased transmissibility and virulence, as well as immune evasion compared to previous Omicron sublineages [22]. BA.2 demonstrated greater transmissibility than BA.1, which permitted its global competition [23]. The presence of several Omicron sublineages in Libya in conjunction with earlier clades, such as 19B and 20B, indicates the evolutionary diversity of SARS-CoV-2 in the country and its integration into the international transmission networks [24]. Ultimately, our results highlight the evolutionary dynamics of SARS-CoV-2 variants in Libya and increase the significance of sustained genomic surveillance in North Africa to detect emerging variants and respond promptly with public health measures.

The complex SARS-CoV-2 clade distribution in Libya could be due to a variety of factors: (1) the limited number of sequences from Libya across the analysis period could limit the representativeness of the data obtained [25]. (2) Variation in sampling protocols and public health management plans might be associated with discrete clade introduction and propagation relative to previously described examples in other Middle Eastern countries [26]. In our genomic analyses, we also reported several key amino acid mutations carried by SARS-CoV-2 Libyan isolates. The most prominent amino acid mutations were D614G, P314L, E484K, and F486V, which have been previously indicated to enhance transmissibility, replication, and immune escape [9][10][12]. The D614G mutation was ubiquitous in clade 22C sequences. Indeed, the D614G was reported early in 2020 and quickly spread around the world by mid-2020, and it served as a conserved hallmark in almost all modern variants [27] [28]. Despite D614G residing outside of the receptor-binding domain (RBD) of the spike protein, it indirectly alters the structural relationship between S1 and S2, allowing the RBD to be in an “open” form that is easily bound by the ACE2 receptor [29] [27]. This structural shift increases the entry of the viral particle and enhances infectivity. Interestingly, an in silico structural study showed that although D614G decreases protein stability, it increases viral transmissibility and adaptability [30]. The second most prominent mutation identified in our study was P314L (P323L), which was present in every sequence assigned to clade 21L.

P314L is located in ORF1b encoding the RNA-dependent RNA polymerase (RdRp), which is an essential component of the viral replication-transcription complex [31]. There is evidence from functional and structural studies that suggests that P314L modifies RdRp and could increase its functional capacity and induce a higher replication efficiency with potential association with disease severity [32]. Notably, P314L typically occurs with D614G, a mutation associated with increased transmissibility and severity [9] [33]. The recurrence of P314L and D614G in Libyan strains reflects a global pattern, suggesting that specific combinations of mutations might work synergistically to enhance viral virulence. Alongside replication- and transmission-enhancing mutations, we observed immune escape-associated substitutions. E484K was identified in Eta variants, whereas Omicron sequences contained F486V [10] [11] [34]. E484K is located within the receptor binding domain (RBD) and might restrict recognition by neutralizing antibodies and monoclonal antibodies, and might improve ACE2 binding affinity and infectivity [10,11]. Similarly, Omicron contained the F486V mutation, which was also shown to decrease the neutralization efficacy of therapeutic monoclonal antibodies [34]. These immune escape mutations demonstrate the ongoing viral adaptation under immune selection. Recurrent mutations were also reported in non-spike regions, including C241T in the 5′ untranslated region (UTR), C884T, T670G, and C1627T in ORF1ab. The C241T mutation was present broadly in our analyzed dataset, and this was noted by an earlier international study, which indicated the dominant occurrence of the C241T mutation in SARS-CoV-2 in the examined lineages [35]. Moreover, the results of another dynamic study have proposed that a mutation in the 5’ UTR region could increase viral replication by altering RNA secondary structures and thus, the interaction with host transcriptional factor [36]. Our genetic data analysis indicates the presence of various ORF1ab substitutions, which could contribute to the observed genetic diversity. However, it is unknown if these mutations could have any

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functional impact. Certainly, some mutations in ORF1ab, for example, P227L, G671S, and P323L, have shown a significant influence on viral replication, changing RNA-dependent RNA polymerase activity [31,32]. Our work emphasizes the need for functional characterization of these ORF1ab variants in order to understand their potential role in viral evolution and adaptation.

All analyzed sequences complied with a comprehensive quality control process, with less than 1% missing data, and absence of frameshifts or premature stop codons, ensuring reliable lineage assignment and mutational tracking. This is in accordance with the stringent quality control protocols advocated by major genomic surveillance organizations, and was necessary for accurate variant assignment [37]. The founder mutations found in our dataset not only confirmed the clade assignments but also were representative of the genetic signatures of the circulating lineages. Clade 21D (Eta) included mutations such as C241T, C884T, C1498T, and ORF1ab: P314L and S: E484K, which have all been highlighted to allow for immune escape and increased infectivity [10] [11] [32] [35]. In addition, Clade 22B (Omicron BA.5) included T670G, C1627T, S: F486V, and ORF1ab: T1001I, which have been identified globally as signature mutations and previously established as facilitating increased transmissibility and immune escape [34]. These divergent mutation profiles illustrate that there were different lineages co-circulating within Libya, and provide an evident picture of the utility of founder mutations for tracking variant spread. Together, these findings illustrate the global and national landscape of SARS-CoV-2 evolution.

Limitations

The current retrospective study has several limitations, despite providing valuable results. Firstly, the studied dataset was relatively limited, with only 30 sequences, which constrains the full scope of genetic variety for SARS-CoV-2 in Libya. Secondly, the thirty sequences were retrieved from a public database (NCBI), which might make the collection strategy non-representative in the context of the geographical aspect. Finally, the descriptive nature of this study and the absence of statistical modelling and clinical relations with patient outcomes could interfere with interpreting the main findings and conclusions of this study.

Conclusion

This descriptive and retrospective study demonstrates a heterogeneous distribution of SARS-CoV-2 clades in Libya, with clade Eta (21D) being predominant, followed by BA.5 Omicron (22B) and rare occurrences of 21L and 22C. Mutations of interest, including D614G, P314L, E484K, and F486V, were also reported, allowing the virus to evolve to become more transmissible and more evasive to immunity. In addition, recurrence of mutations in the non-spike region suggests ongoing diversification of the virus. The identified clades and their related mutational profiles reflected local evolutionary specification. Importantly, the detection of the Eta clade in Libya, despite its rare occurrence globally, indicates that SARS-CoV-2 has distinctly different evolutionary paths in Libya compared to neighboring countries. Our findings underscore the increased need for localized, high-quality genomic surveillance to capture differences in epidemic dynamics, guide public health measures, and improve preparedness strategies.

Conflicts Of Interest. Nil

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