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Isolation and Characterization of SARS-CoV-2 in Kenya

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Abstract

The discovery of Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) in Wuhan, Hubei province, China, in December 2019 raised global health warnings. Quickly, in 2020, the virus crossed borders and infected individuals across the world, evolving into the COVID-19 pandemic. Notably, early signs of the virus's existence were observed in various countries before the initial outbreak in Wuhan. As of 12th of April, the respiratory disease had infected over 762 million people worldwide, with over 6.8 million deaths recorded. This has led scientists to focus their efforts on understanding the virus to develop effective means to diagnose, treat, prevent, and control this pandemic. One of the areas of focus is the isolation of this virus, which plays a crucial role in understanding the viral dynamics in the laboratory. In this study, we report the isolation and detection of locally circulating SARS-CoV-2 in Kenya. The isolates were cultured on Vero Cercopithecus cell line (CCL-81) cells, RNA extraction was conducted from the supernatants, and reverse transcriptase-polymerase chain reaction (RT-PCR). Genome sequencing was done to profile the strains phylogenetically and identify novel and previously reported mutations. Vero CCL-81 cells were able to support the growth of SARS-CoV-2 *in vitro*, and mutations were detected from the two isolates sequenced (001 and 002). Genome sequencing revealed the circulation of two isolates that share a close relationship with the Benin isolate with the D614G common mutation identified along the S protein. These virus

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isolates will be expanded and made available to the Kenya Ministry of Health and other research institutions to advance SARS-CoV-2 research in Kenya and the region.

Keywords

SARS-CoV-2, COVID-19, Whole Genome Sequencing, Phylogenetic Analysis, Nucleotide Substitutions, Amino Acid Changes

1. Introduction

The coronaviruses (*coronaviridae)* were first recognized as a new family of viruses in 1968. They are enveloped, positive-sense-stranded ribonucleic acid (RNA) viruses with a genome of 26 - 30 kb, with the largest genome among all the known RNA viruses [1]. The name of these viruses is derived from their morphology with spike proteins on their surface that appear like a crown shape [2]. Phylogenetically, coronaviruses are classified into four genera: Alphacoronaviruses, Betacoronaviruses, Gammacoronaviruses, and Deltacoronaviruses. There are four human coronaviruses, namely, 229E, Netherland 63 (NL63), Organ culture 43 (OC43), and Hong Kong University 1 (HKU1), which infect the upper respiratory tract and cause mild symptoms. There are three other coronaviruses of zoonotic origin that infect the lower respiratory tract of humans and can cause severe respiratory illness and lead to fatalities. These are severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV) [3] [4], and SARS-CoV-2.

However, recently, a new coronavirus strain emerged in Wuhan, China, that has caused a worldwide pandemic that was later named SARS-CoV-2 by the International Committee on Taxonomy of Viruses [5]. Sequence and phylogenetic analysis of various published strains show close similarity (99% homology) to the wild-type SARS-CoV strain [6]. Research is still underway to understand the aetiology and pathophysiology of the virus.

As of 12th April 2023, COVID-19 had infected over 762 million individuals with over 6.8 million deaths globally, of which 9.5 million cases and over 175,000 deaths occurred in Africa [7]. Almost all the regions across the world have recorded COVID-19 cases. However, some countries such as Turkmenistan have not yet registered any cases [8]. The previous outbreaks of coronaviruses, including SARS and MERS, affected over 26 and 27 nations, [9] with morbidity of 8000 and 2519 for SARS and MERS, respectively [10]. Compared to other pandemics, COVID-19 has caused higher morbidities and an overall lower case fatality ratio (CFR) as of 16th December 2022 [11]. Comorbidities such as diabetes, cancer, and hypertension have been linked to high mortalities due to COVID-19. On the other hand, Africa is known to be high in morbidities of infectious diseases such as HIV/AIDS and Malaria [12] [13] [14]. Recent studies have been able to link such morbidities with COVID-19 co-infection and severe disease [15] [16].

SARS-CoV-2 isolation and culture were first conducted in South Korea, where in February 2020, nasopharyngeal (NP) and oropharyngeal (OP) samples were isolated from confirmed cases of COVID-19 patients, isolation and replication were confirmed through viral culture and gene sequencing [17]. In June 2020, in the same country, serum, urine, and stool specimens were used to isolate the SARS-CoV-2, where the presence of the virus was evaluated using real-time RT-PCR [18]. In July 2022, a study was conducted in Kenya on the isolation and characterization of SARS-CoV-2 from wastewater where they found out SARS-CoV-2 variants belonging to Alpha and Delta lineages circulated in Nairobi, Kenya [19]. Our study aims to describe the isolation, culture and characterization of SARS-CoV-2 using nasopharyngeal and oropharyngeal swab samples collected from 14 patients in 4 different counties in Kenya (Nakuru, Busia, Taita Taveta, and Machakos Counties).

2. Materials and Methods

2.1. Specimen Collection

NP and OP swabs were collected as described [20] from symptomatic patients reported to health facilities in Kenya in July 2020, and placed in a virus transport medium (VTM). To maintain confidentiality, the samples were assigned unique identities and delinked from the patient information. The samples were transported to KEMRI in cold packs and stored at the Sample Management and Receiving Facility (SMRF) at −80˚C pending processing. The handling and processing of the samples were done at the KEMRI biosafety level 3 laboratory, where analysis was performed as described in the US Centers for Disease Control (CDC) guidelines [21].

2.2. Isolation of SARS-CoV-2 from Clinical Samples

Isolation and propagation of high titers of infectious SARS-CoV-2 were done in a biosafety level 3 laboratory by qualified staff using a modified method by Tastan and colleagues [22]. Following confirmation of the virus by conventional RT-PCR, low passage Vero CCL-81 cells were cultured in from the cell bank were thawed into Minimum Essential Medium (MEM, Life Technologies Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) and 2% Penicillin-Streptomycin-Amphotericin solution (Gibco). The frozen samples were thawed on ice, and a 10× dilution was prepared in single-strength MEM. 100 µl of the diluted virus was inoculated into wells of a 96-well culture plate of Vero CCL-81 cells. Upon confirmation of CPE under an inverted microscope, the contents of the wells were sequentially propagated in culture plates and flasks (NUNC, Roskilde, Denmark) containing 90% confluent monolayers of Vero cells. In all cases, plates and flasks were maintained in a humidified 5% CO_2 atmosphere in an incubator at 37˚C. Cells were monitored daily for CPE and at 85% of CPE; the intracellular fluid (ICF) from the 75 cm^2 flask was harvested by centrifuging for 10 minutes at 4˚C. The supernatant was aliquoted and kept at −80˚C as seed virus stock.

2.3. SARS-CoV-2 Viral Titer Determination

Plaque assays were done based on SARS-CoV-2 and MERS-CoV protocols [23] with a few modifications. Vero CCL-81 cells were seeded in 6-well culture plates to attain a 90% cell confluence before inoculation with 200 µl of seed virus stock. After overlaying the infected cells with agarose gel, the plates were incubated at 37˚C in a 5% carbon dioxide gas atmosphere for 3 - 4 days until plaques were evident. Staining was done with neutral red dye to visualize the plaques, and after overnight incubation, the plaques were counted and determined using the formula: Titer (pfu/ml) = number of plaques × dilution factor × 1/volume of virus added to cells in a well (ml).

2.4. Viral RNA Extraction

Viral RNA was extracted from the supernatants using the viral RNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and temporarily kept at −20˚C awaiting reverse transcription.

2.5. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Reverse transcription polymerase chain reaction was done as described [24]. Primers targeting the RdRp gene forward (5'-CAAGTGGGGTAAGGCTAGAC TTT-3') and reverse (5'-ACTTAGGATAATCCCAACCCAT-3') with an amplicon size of 344 bp and S gene forward (5'-CCTACTAAATTAAATGATCTC TGCTTTACT-3') and reverse (5'-CAAGCTATAACGCAGCCTGTA-3') with an amplicon size of 158 bp were used in this assay [24]. Reverse-transcription was carried out at 50 ˚C for 30 minutes followed by 95 ˚C for 15 minutes, 50 cycles of 95 ˚C for 60 s, 55 ˚C for 60 s and 1 step at 72 ˚C for 10 minutes. Positive and negative controls were included in all assays. For the no template control, 5 µl of nuclease-free water was added in place of the RNA template. Likewise, 5 µl of RNA with a known Ct value was added in place of template RNA to serve as the positive control. Cycles were run on Applied Biosystems ABI 7500 Fast instrument. Finally, gel electrophoresis was conducted using 2% agarose gel at 100 V for 45 minutes, stained for one hour and visualized under a UV-transilluminator (Maestrogen Inc, Taiwan, China).

2.6. Whole Genome Sequencing of the RT-PCR Product

A total of 50 ng cleaned-up PCR product was repaired using NEBNext Ultra II End repair/dA- tailing reagent and barcoded using NEBNext Ultra II Ligation Master Mix (New England Biolabs Inc. Massachusets, USA), following manufacturer's guidelines. The barcoded DNA was pooled and purified using AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, USA) and quantified using a fluorometer (DeNovix Inc. Wilmington, USA). At least 2 ng/ul of the DNA libraries were loaded on a flow cell and sequenced on a MinION (Oxford Nanopore Technologies, Oxford, United Kingdom), which sequences long reads of the ssDNA molecule with cartridges containing 2048 nanopores arranged in 512 channels. Leader

and hairpin adapters were used to prepare dsDNA that was recognized by specific signals generated by the apurinic and/or apyrimidinic sites in the hairpin. Sequence consensus was built following a successful reading of the DNA strands.

Raw read sequences from the Oxford Nanopore MinION sequencing platform were evaluated for quality based on FastQc reports and deposited in the NCBI SRA database with accession PRJNA825709. The quality threshold was set at 20, and a minimum length of 150 was allowed. A trimmomatic tool [25] was used to remove adapter sequences, followed by a post-trimming quality assessment based on the FastQC report. We used SPADES [26] to assemble raw sequences with reference genome retrieved from the SARS-CoV-2 RefSeq database: https://ftp.ncbi.nlm.nih.gov/refseq/release/viral/; March 10th version. Raw sequences were deposited in the NCBI's Sequence Retrieval Archive (SRA) under accessions SRS12585595 and SRS12585594. The sequences were identified using nucleotide Basic Local Alignment Search Tool-BLAST [27]. Upon confirming the sequence, we performed cluster analysis of the isolates using the Nextstrain/Nextclade v1.14.0 [28] server. Equally, mutations in amino acid residues were conducted using Nextclade. Sequence alignment and phylogenetic comparisons were achieved using MUSCLE [29] and MEGAX [30].

3. Results

3.1. Isolation of SARS-CoV-2 in Culture

Three of the four clinical samples indicated cytopathic effect (CPE) in Vero CCL-81 cells (001, 003, and 004) three days post infection (**Figure 1** and **Figure 2**) following the initial infection and infectious culture fluids (ICFs) were frozen for further analysis. Sample 002 did not show CPE and ICF was kept for blind passaging. Sample 004 ICF was selected for sequencing.

3.2. Quantification of SARS-CoV-2 by Plaque Assay

The isolated SARS-CoV-2 formed distinctly visible plaques, and the viral titer was determined as $1{\cdot}65 \times 10^5$ pfu/ml.

3.3. Conventional RT-PCR

A multiplex RT-PCR was conducted on 14 ICFs extracted and isolated from the 4 clinical samples that indicated CPEs targeting the RdRp (344 bp) and S (158 bp) genes. Gel results were as shown below (**Figure 3** and **Figure 4**).

Out of the 14 samples used in this study, 13 appeared positive for SARS-CoV-2 RdRp and S genes (92.9%).

3.4. Relationship between the Local Isolates and the Global SARS-CoV-2

3.4.1. Whole-Genome Sequencing and Phylogenetic Analysis

We successfully assembled the whole genomes and confirmed the identity of SARS-CoV-2 virus isolates from our cultured isolates. The length of the assembled

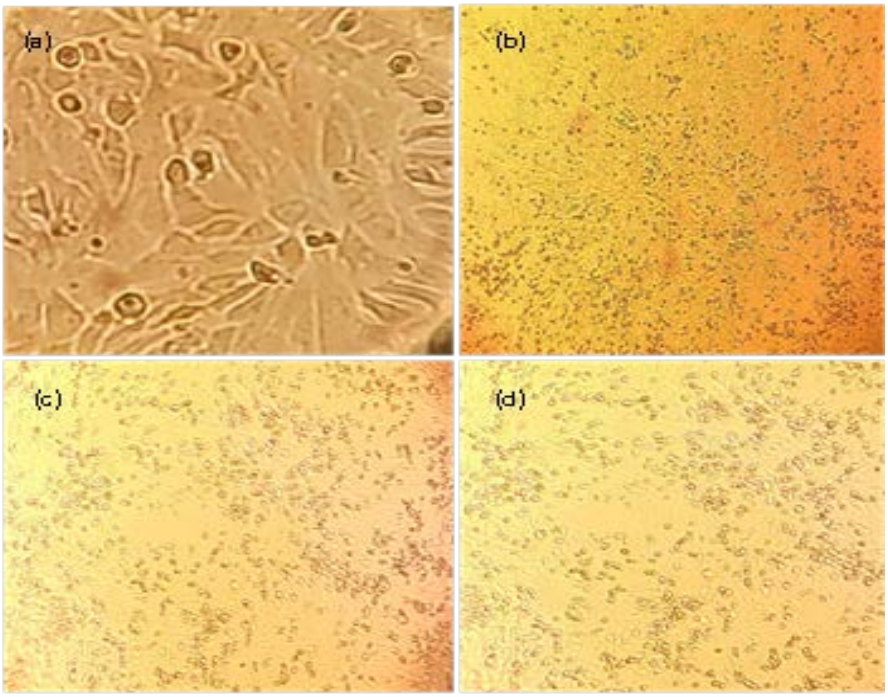

Figure 1. Phase contrast images of SARS-CoV-2 replication and cytopathic effects (CPE) in Vero CCL-81 cell lines at 10X magnification. (a) Negative control cells inoculated with culture media. (b) Vero cells 2 days post infection. (c) Vero cells rounding up and floating in suspension 3 days post infection. (d) Cells rounding up 4 days post infection and CPE at 85%.

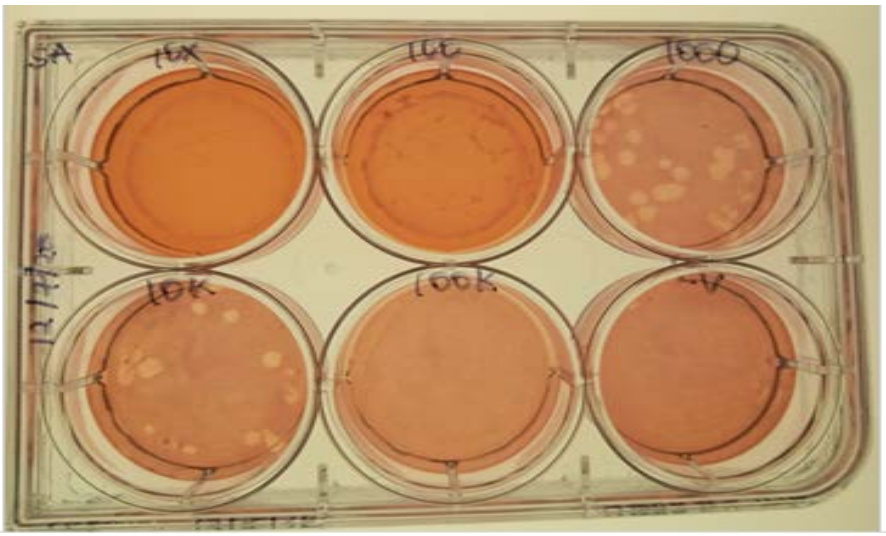

Figure 2. Showing plaque formation of SARS-CoV-2 virus from passage 2 stocks at 3 days' post infection on Vero cells CCL-81 cell lines

genomes from isolates 001 and 002 were 29,829 bp and 29,903 bp, respectively. This represents 100% coverage of the reference genome. Comparisons of the sequences from this study to previous isolates from Kenya and the rest of the world in databases such as Nextrain-GISAID [31] show apparent similarity, particularly to samples from Africa. We clustered the sequences based on ancestral

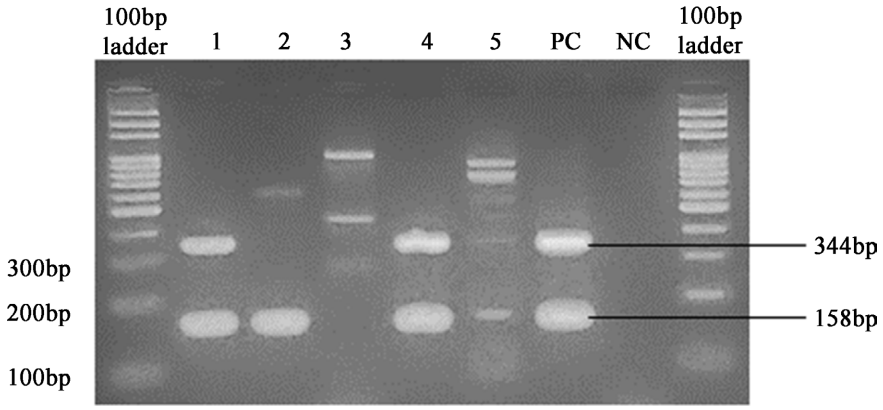

Figure 3. Showing 5 PCR products run on 2% agarose gel. Lanes 1 - 5 contained a sample. Clear bands targeting the RdRp (344 bp) and S (158 bp) genes can be seen for samples 1 and 4. Sample 2 was positive for the S gene. PC and NC are the positive and negative controls respectively.

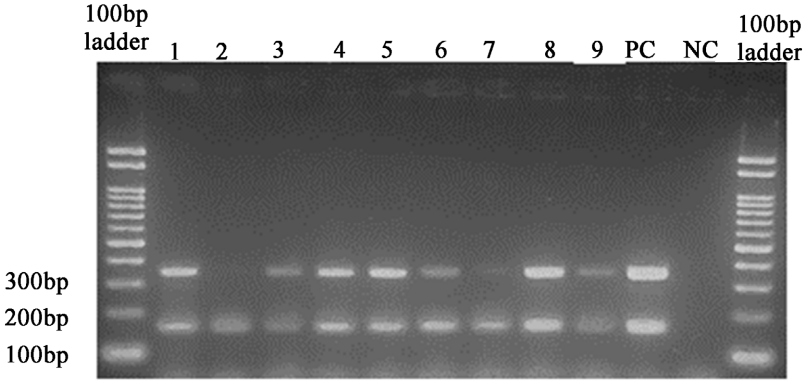

Figure 4. Showing 9 PCR products run on 2% agarose gel.

similarity to confirm the clades where our isolates belong and further confirm the identity of our cultures. While our isolates clustered closely to other Kenya SARS-CoV-2 isolates, they did not fall entirely on the Kenyan clade but under a sub-clade comprising Benin isolates (**Figure 5**).

Further classification based on the Next clade, analysis shows the strains to belong to the clade 20C, characterized by mutations [S: D80Y; N: S186Y; N: D377Y; ORF1a: T945I; ORF1a: T1567I; ORF1a: Q3346K; ORF1a: V3475F; ORF1a: M3862I; ORF1b: P255T; ORF7a: R80I] thought to have a Western USA origin (**Figure 6**).

3.4.2. Nucleotide Substitutions and Amino Acid Changes

To confirm substitutions at the protein level and follow up on the Nextclade classification of the isolates to the clade 20C, we aligned the sequences and profile mutations as shown in **Table S1**. While variations across the entire genome, pronounced differences in nucleotide sequences are evident in the S protein-coding sequences (CDS) (Supplementary **Figure S1**). However, most of these are silent mutations, as we could identify only seven mutations in each sequence (**Table S1** and Supplementary **Figure S2**).

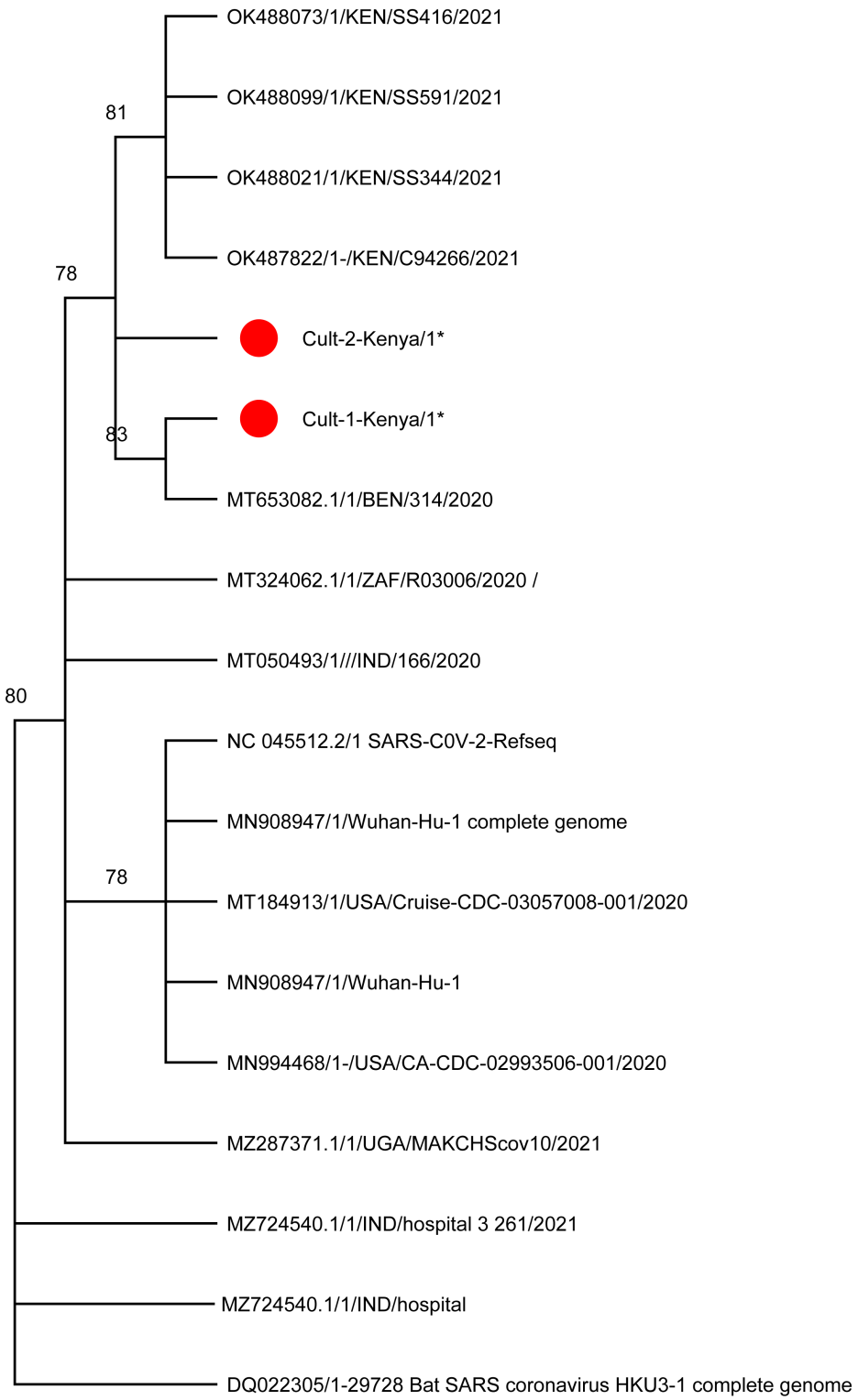

Figure 5. Phylogeny relationship between the two Kenya isolates and sequences from databases based on Neighbor-Joining parsimony.The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1, in which the initial trees were obtained by the random addition of sequences (10 replicates).

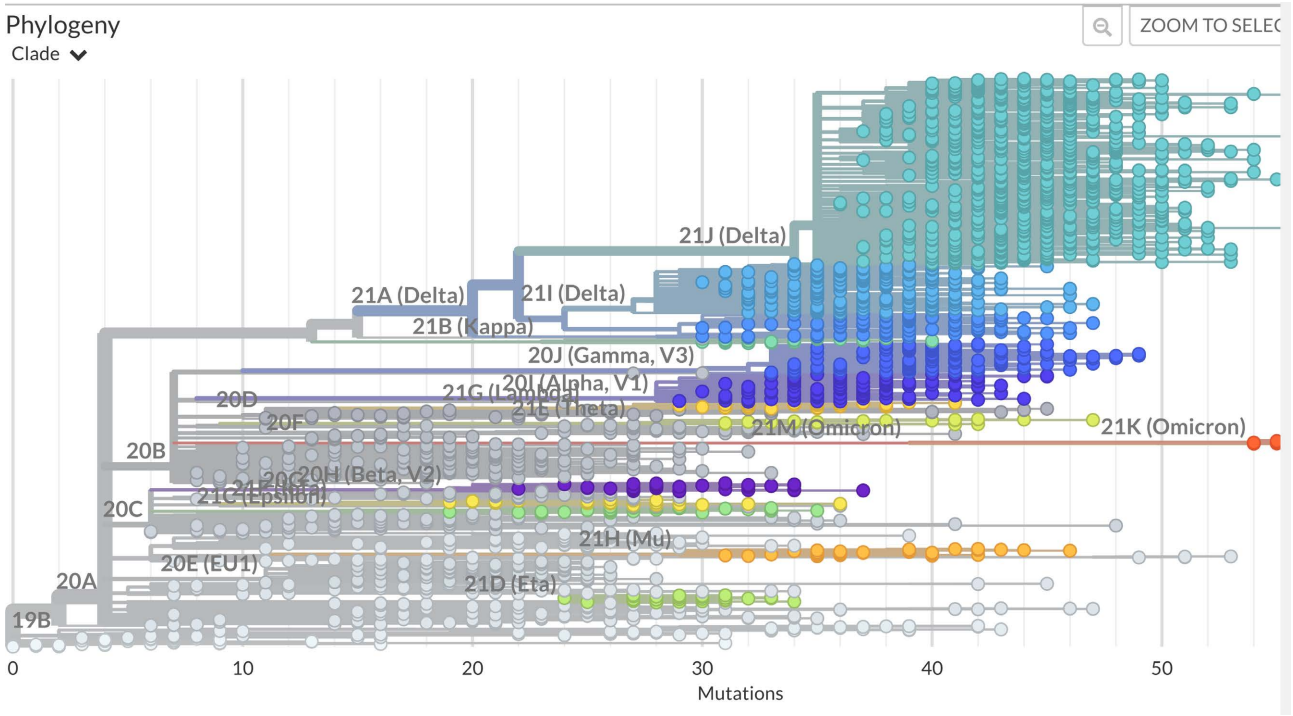

Figure 6. Phylogenetic tree built using Nextclade online software (https://clades.nextstrain.org/tree (accessed on April 12th, 2022) and visualized using Auspice online tool (https://auspice.us/ (accessed on April 12th, 2022). Highlighted in red is the clade classification of sequences from this study.

4. Discussion

In our study, the SARS-CoV-2 virus was successfully isolated in Vero CCL-81 cells, reportedly successfully culturing the virus [32] [33]. NP and OP specimens of patients in Kenya were used for the virus isolation. The two different coronaviruses that occurred in Asia, SARS-CoV and MERS-CoV, were found to grow well on Vero CCL-81 [34]. In our analysis, SARS-CoV-2 replication was evident based on the observable CPE and subsequent plaque formation, implying the importance of the cells in-vitro study of the virus. A comparative study was done by Harcourt *et al.,* (2020) [35], where they isolated and characterized SARS-CoV-2 from the first COVID-19 patient in the United States using both VeroE6 and CCL-81 cells. Both cells were observed to support the amplification and quantification of the virus. Our study is consistent with the Korean isolate, where the CPE occurred on Vero cell cultures after passaging on the third day [36]. Isolation and characterization of SARS-CoV-2 will be helpful in studying the immune response of COVID-19 patients to monitor their ability to block viral infections. Furthermore, the local isolates can play a big role in antiviral research through locally available antiviral medications to treat or prevent COVID-19. Another area of much focus is vaccine development as pharmaceutical companies and research institutions are in a race to develop robust vaccines that effectively end the pandemic. Therefore, as part of the global effort, research in the African continent such as Kenya to try to understand more about the virus pathogenesis is very essential for better future diagnostics and vaccine de-

velopment.

After establishing an immense pattern of infection of SARS-CoV-2 on Vero CCL-81 cells, we extracted RNA from 14 ICFs and carried out conventional multiplex RT-PCR targeting the RdRp and S genes. RT-PCR has been considered to be the gold standard in SARS-CoV-2 identification; however, different testing kits give contrasting results based on specificity and sensitivity [37]. The virus consists of the Spike (S), Membrane (M), Envelope (E), Nucleocapsid (N), and the RNA-dependent RNA polymerase (RdRp) proteins. Most of the RT-PCRs target the S gene due to its affinity and binding nature to the angiotensin-converting enzyme 2 (ACE-2) receptors. Despite the positive outcome, previous studies have estimated the sensitivity of conventional RT-PCR to be between 70% - 98% and specificity approximated at 95% [38] [39]. The variation is due to the fact that the viral load for an individual changes over the duration of the infection [40]. It was also shown that the diversity of SARS-CoV-2 can affect the outcome of an RT-PCR analysis [41]. Our study was successful in targeting both genes showing they can be effective in detecting the virus from clinical samples.

SARS-CoV-2 continues to affect countries of the world. Studying in detail the virus genome will open up more features in tracing the virus source path and source of infection [42]. In this study, several genomes were aligned together with our two isolates for characterization based on phylogenetic analysis. The results show that our two isolates share a close relationship with the Benin isolate while the Nextclade analysis showed direct linkage to the Western USA isolate. The nucleotide sequences and their resultant amino acid (AA) composition were further investigated for any possible mutations. Most of the SARS-CoV-2 mutation studies have been done along with the S and N proteins. D614G mutation along the S protein has been reported from previous analysis, seeming to be a predominant one [43] [44], enhancing the binding affinity of SARS-CoV-2 to the ACE2 receptors [45]. Both isolates had this mutation at the S protein, with recent studies showing the AA change to aid in faster viral transmission [46]. All nucleotide substitutions were common in the isolates across the different genes despite most of them giving silent mutations. An R195K AA change in the N protein was reported in this study though from a different position from the one found in this study (R203K) [47], which may be significant in blocking an immune response through antibody production among infected patients. C-T single nucleotide polymorphism (SNP) was seen to occur along the open reading frame-1a (ORF1a). Previous studies have shown that the C-T SNP at position 28144 led to a Serine-Leucine amino acid change, thereby elucidating a higher transmission rate [48]. In our study, a T265I and S2558Y AA substitutions were found to occur along the ORF1a.

So far, a number of variants with multiple mutations have been discovered since the emergence of the pandemic. This shows that the SARS-CoV-2 virus continues to evolve so as to evade the host defense system despite efforts to eliminate it through the use of currently developed vaccines and antivirals. Our

study was successful in deciphering the evolution of the SARS-CoV-2 within the Kenyan population through phylogeny and mutational analysis.

5. Conclusion

This study showed that Vero CCL-81 cells could support SARS-CoV-2 virus isolation and characterization through the formation of viral plaques. Detection of SARS-CoV-2 targeting the S and RdRp genes was effective. However, a limitation of this study was that the isolates cultured were from samples collected in July, 2020, when the pandemic emerged, with very few strains reported. As we are aware, the virus has dynamically evolved; hence future studies should focus on recent samples to sequence viral genomes from different isolates collected in different regions to increase the probability of identifying multiple strains circulating in Kenya and even possible novel mutations, which will also offer a road map in the development of diagnostics and vaccines to control the spread of the disease. Another significant area of study could be on co-infections of SARS-CoV-2 with other endemic diseases such as HIV/AIDS and malaria that have continued to affect the Kenyan population for a long time so as to depict disease severity due to co-infection within a human host. A study can also be conducted to investigate new strains in circulation in Kenya.

Funding

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Institutional Review Board Statement

The Scientific and Ethics Review Unit (SERU-KEMRI) approved this study under protocol number KEMRI/SERU/CBRD/210/4027. Vero CCL-81 cell line was obtained from the Kenya Medical Research Institute cell line bank.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from patients to publish this paper.

Data Availability Statement

The data presented in this study are available in form of figures, supplementary figures, and tables provided within the article.

Author Contributions

Conceptualization, S.M.N.; Methodology, S.M.N., A.M., R.M.I., M.M.M and C.W.N.; Software, R.T., V.R.; Validation, S.M.N.; Formal Analysis, S.M.N., A.M., R.M.I.; Investigation, A.M., M.M.M., R.M.I., C.W.N., P.K.R.,T.T.N., J.J.Y., A.W.M., and J.H.K.; Resources, S.M.N.; Data Curation, S.M.N., A.M., R.T., and R.M.I.; Writing-Original Draft Preparation, S.M.N., A.M., R.M.I., Writ-

ing-Review and Editing, S.M.N., R.T., V.R., and R.M.I.; Visualization, S.M.N.; Supervision, S.M.N.; Project Administration, S.M.N.; Funding Acquisition, S.M.N., and J.H.K.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Appendix

Appendix 1

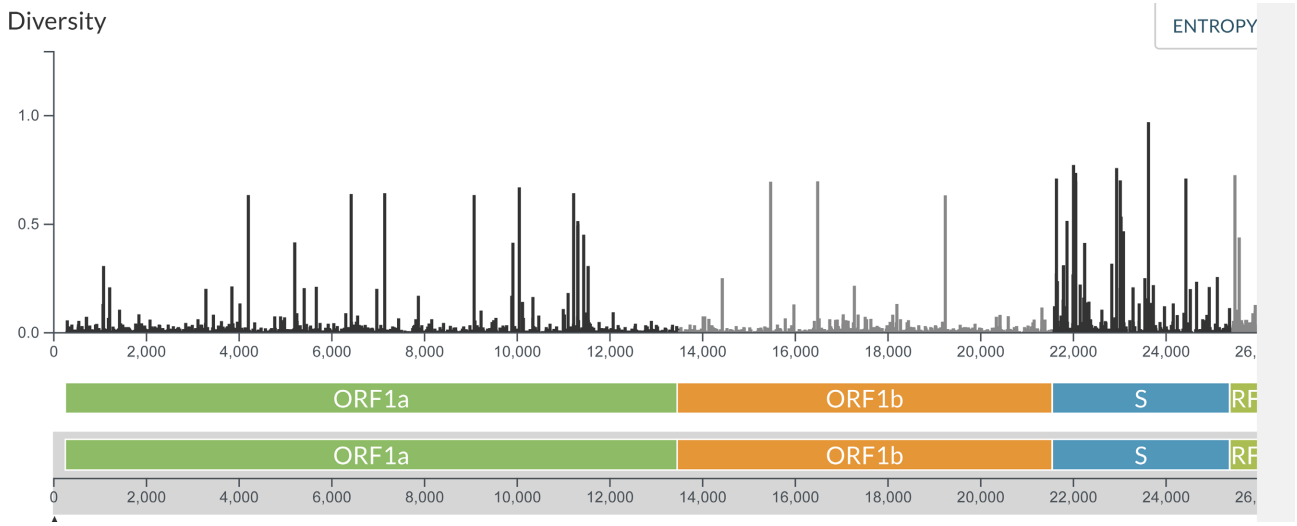

Figure S1. Combined overview for genetic diversity of the two sequences from cultured isolates. We used the Nextstrain server (https://clades.nextstrain.org/tree (accessed on April 12th, 2022) to generate the diversity bars and visualize using Auspice online tool (https://auspice.us/ (accessed on April 12th, 2022). The CDS representative of sequence one is illustrated alongside the reference SARS-Cov-2 genome.

Appendix 2

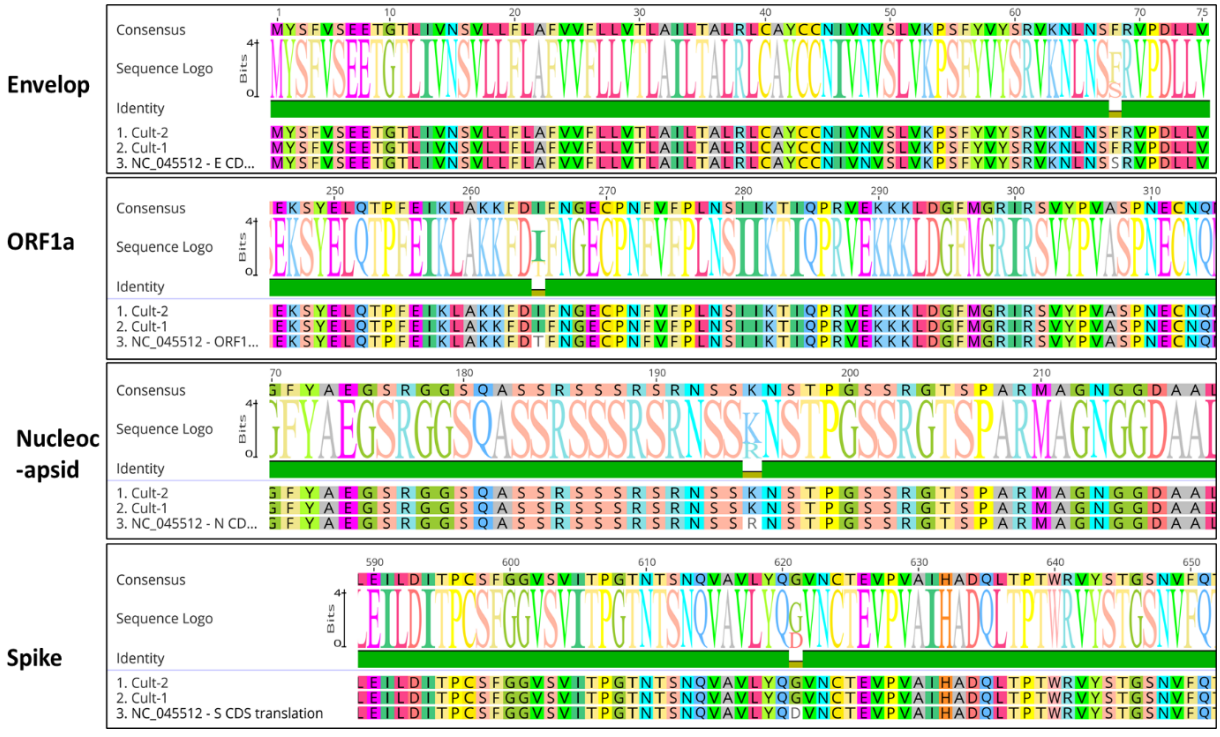

Figure S2. Sequence alignments. This study's alignment of isolates 001 and 002 against the reference SARS-CoV-2 genome. Emphasis is on the regions with amino acid substitutions in the envelope, nucleocapsid, spike, and ORF1a CDSs. A gap in the identity bar indicates substitution, and the height of the logo corresponds to conservation at a given amino acid residue. Whole alignments at a given CDS are provided as separate files.

Table S1. Mutations (substitutions) in amino acid sequences from the cultured isolates.

Isolate	CDS	Amino acid substitution	Nucleotide mutations
Isolate 001	E	E:S68F	G7C, G8T, A12T, A14T, C16G, C20G, A27T, C28T, A29C, A30C, C7938A, G7940T, C8320T, C13168T, C26447T, G28857A, A29876T, A29878C
	N	N:R195K	
	ORF1a	ORF1a:T265I; ORF1a:S2558Y	
	ORF1b	ORF1b:P314L	
	ORF3a	ORF3a:Q57H	
	S	S:D614G	
Isolate 002	E	E:S68F	A4G, A5T, G7C, G8T, A12T, A14T, C16G, C20G, A27T, C28T, A29C, A30C, C7938A, G7940T, C8320T, C13168T, C26447T, G28857A, A29876T, A29878C
	N	N:R195K	
	ORF1a	ORF1a:T265I; ORF1a:S2558Y	
	ORF1b	ORF1b:P314L	
	ORF3a	ORF3a:Q57H	
	s	S:D614G	

Note: E: Envelope; N: Nucleocapsid; S:Spike protein.