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SARS-CoV-2 variant typing using real-time reverse transcription-polymerase chain reaction–based assays in Addis Ababa, Ethiopia

Wodneh G/meskel[1], Kassu Desta[1], Regasa Diriba[1], Mahlet Belachew[2], Martin Evans[3], Vlademir Cantarelli[3], Maritza Urrego[3], Abay Sisay[1], Atsbeha Gebreegziabxier[4], Adugna Abera[2,*]

[1] Department of Medical Laboratory Sciences, College of Health Science, Addis Ababa University, P.O.Box 1176, Addis Ababa, Ethiopia
[2] Malaria and Neglected Tropical Diseases Research Team, Ethiopian Public Health Institute, Addis Ababa, Ethiopia
[3] Global Public Health Programs, American Society for Microbiology, Washington, USA
[4] HIV and TB Research Directorate, Ethiopian Public Health Institute, Addis Ababa, Ethiopia

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ABSTRACT

Objectives: This study aimed to determine the SARS-CoV-2 variants in the first four COVID-19 waves using polymerase chain reaction (PCR)–based variant detection in Addis Ababa, Ethiopia.

Methods: A cross-sectional study was conducted using repository nasopharyngeal samples stored at the Ethiopian Public Health Institute COVID-19 testing laboratory. Stored positive samples were randomly selected from the first four waves based on their sample collection date. A total of 641 nasopharyngeal samples were selected and re-tested for SARS-CoV-2. RNA was extracted using nucleic acid purification instrument. Then, SARS-CoV-2 detection was carried out using 10 μl RNA and 20 μl reverse transcription-PCR fluorescent mix. Cycle threshold values <38 were considered positive.

Results: A total of 374 samples qualified for B.1.617 Lineage and six spike gene mutation variant typing kits. The variant typing kits identified 267 (71.4%) from the total qualifying samples. Alpha, Beta, Delta, and Omicron were dominantly identified variants from waves I, II, III, and IV, respectively. From the total identified positive study samples, 243 of 267 (91%) of variants identified from samples had cycle threshold values <30.

Conclusions: The study data demonstrated that reverse transcription-PCR–based variant typing can provide additional screening opportunities where sequencing opportunity is inaccessible. The assays could be implemented in laboratories performing SARS-CoV-2 molecular testing.

Introduction

SARS-CoV-2 is a single-stranded positive RNA that possesses 30,000 genomes. Mainly, its genome encodes the spike (S), envelope, membrane protein, nucleoprotein of a structural protein, and 16 nonstructural proteins. SARS-CoV-2, similar to other RNA viruses prone to genetic change, is a mutation that originates from erroneous replication during genetic recombination [1]. Variants develop through S gene mutation, either by substitutions, deletions, insertions, or single-nucleotide changes in the genome.

In the mid-2020s, Alpha, the first variant of concern, was reported in the United Kingdom [2]. Subsequently, different variants have been identified in different parts of the world, some being considered variants of concern (VOCs) and variants of interest (VOIs) given their impact on public health [2,3].

As of May 18, 2023, Ethiopia had experienced five successive COVID-19 epidemic waves. The first wave was observed during World Health Organization (WHO) epidemic weeks 32-36 of 2020. The second wave followed a few months in WHO epidemic weeks 9-17 of 2021 [4]. The third wave was observed during epidemic week 33-39 of 2021, the fourth wave was in WHO epidemic week 46 of 2021 to week 3 of 2022, and the last wave was in WHO epidemic week 20-28 of 2022 (Supplement 4).

Next-generation sequencing (NGS) is the recommended reference method for identifying SARS-CoV-2 variants on a global scale. However, it is high-tech and cost-limited in its availability, making it unrealistic for the timely detection of variants in a public health response. Moreover, it requires a relatively technical expertise and a longer data processing time. Accordingly, it is difficult to implement NGS in diagnostic laboratories, especially in resource-poor countries. Therefore, alterna-

* Corresponding author: Tel: +251911883483.
E-mail address: adugnabe@yahoo.com (A. Abera).

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tive screening methods are desirable for early detection and monitoring of the spread of specific variants [1,2].

To improve the timely detection of variants, WHO recommends all countries perform genomic sequences for at least 1% of their positive cases [2,3]. As of June 8, 2023, more than 15.7 million SARS-CoV-2 sequences were deposited in the Global Initiative on Sharing All Influenza Data and publicly shared. From this, Ethiopia has sequenced a very small number of variants from each wave (https://gisaid.org/). As a result, the variants that caused the four national COVID-19 waves were not well identified.

Thus, this study aimed to identify the variants responsible for the first four national COVID-19 waves, determine the distribution of S mutations across the four VOCs, and measure the level of variant-typing polymerase chain reaction (PCR) detection with respect to diagnostic reverse transcription (RT)-PCR cycle threshold (Ct) values.

Material and methods

Ethical considerations

The study protocol was reviewed and approved by the Addis Ababa University College of Health Sciences, Medical Laboratory Sciences departmental research and review committee (Ref. No. MLS/143/22).

Study design and setting

A cross-sectional study was conducted from August to October 2022 at the Ethiopian Public Health Institute's Parasitology COVID-19 testing laboratory [5]. Samples were primarily referred from different subcities of Addis Ababa for COVID-19 suspected cases as part of community screening and patient follow-up. After diagnostic testing, all SARS-CoV-2–positive and 10% of SARS-CoV-2–negative samples were stored at −80°C in the national biobank of the Ethiopia Public Health Institute.

The samples collected from the repository were sorted based on the sample collection date and assigned to specific wave groups. Diagnostic RT-PCR was performed to determine the positive status of the study samples before they were arranged for variant typing by PCR. Ct values <38 defined a positive result. For variant characterization, two variant-typing PCRs (Delta SARS-CoV-2 and six S gene mutant detections) assays were used simultaneously. The number of mutant genes and the type of mutant gene determined the type of variant. The variant-typing PCR positivity was assessed with respect to the diagnostic RT-PCR Ct value. As part of the quality control, we did sub-sampled NGS for Delta variant assay in which all of them were positive for the Delta variant by Oxford Nanopore Technology.

Study samples have been selected by simple random sampling, represented by the four waves. Based on the available qualified specimens, 641 study samples were selected, which correspond to and encompass the first four epidemic waves. From the first wave, a total of 138 samples were selected, whereas 191 study samples were selected from the second wave. In the third and fourth waves, 232 and 80 total study samples were selected for RT-PCR testing, respectively (Table 1).

RNA extraction

All selected nasopharyngeal (NP) samples were extracted on a Bioer NPA-32P instrument (Bior Technology, Zhejiang, China), a high-throughput automated workstation. MagaBio Plus RNA Purification Kit II (Bior Technology, Zhejiang, China) was used for the extraction. All processes were undertaken per the manufacturer's instructions. The 96-well plates were prepared and 300 μL of brief vortex NP specimens were added to columns #1 and #7 for each well. Then, the plate was placed into the instrument, which agitates the plates every 10 seconds for 9 minutes until the extraction is finished. Finally, the 70 μL RNA extract was stored at −80°C for further testing.

RT-PCR detection

A fluorescent RT-PCR (BGI China) commercial kit was used for SARS-CoV-2 detection. The kit targets the ORF1ab gene. Briefly, 10 µl of RNA was added to the respective well containing 20 µl of master mix solution. The amplification reaction was performed in the QuantStudio5 DX real-time PCR system (catalog number A34322, Thermal Fisher Scientific). The cycle conditions were 50°C for 20 minutes and one cycle, 95°C for 10 minutes and one cycle, then 95°C for 15 seconds and 60°C for 30 seconds for 40 cycles. All procedures were performed based on the manufacturer's instructions. A Ct <38 is considered positive for SARS-CoV-2 and ready for variant typing by RT-PCR. After the detection of the SARS-CoV-2 in the tested samples, we determined the variants using Delta SARS-CoV-2 variant detection and the six S gene mutant detection.

Delta SARS-CoV-2 variant identification

Briefly, 10 μL of RNA was added to a 20 μL reaction mixture (BGI PathoGenesis Pharmaceutical Technology Co., Ltd, https://www.bgi.com/us/sars-cov-2-variant-detection/). The kit comprised positive and negative controls; reaction mixes A and B contain sequence-specific primers and fluorescent probes which target the L452R and E484Q genes. Testing was carried out in the following conditions: 50°C for 10 minutes, followed by 1 minute at 95°C, 45 cycles of 95°C for 5 seconds, and 58°C for 15 seconds on the QuantStudio5 thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). The presence or absence of mutant genes is determined by the Ct allele. The ΔCt allele calculated the difference between the Ct allele and the Ct ORF1ab. When the difference in the Ct allele equals or is less than six, it is positive for a particular mutant target gene [6].

The six S gene mutant detections

A total of 10 μL of RNA was added to the reaction mixture of 20 μL (BGI PathoGenesis Pharmaceutical Technology Co., Ltd, https://www.bgi.com/us/sars-cov-2-variant-detection/). The kit comprises positive and blank controls; the reaction mix A contains primers and probes for ORF1ab amplification and internal reference targets N501Y and K417N. Reaction mix B also contains primers and probes for amplification of the A570D, HV69-70del, K417T, and E484K genes. The assay was carried out at 50°C for 10 minutes, followed by 95°C

Table 1
Sample selection and distribution across the four waves, Ethiopia Public Health Institute, 2023.

Wave designation	World Health Organization weeks	Total number of samples selected from respective wave	RT-PCR negative	RT-PCR positive
Wave-1	32 to 36 of 2020	138	68	70
Wave-2	9 to 17 of 2021	191	143	48
Wave-3	33 to 39 of 2021	232	56	176
Wave-4	46 of 2021 to 3 of 2022	80	0	80
Total		**641**	**267**	**374**

RT-PCR, reverse transcription-polymerase chain reaction.

for 1 minute, 45 cycles of 95°C for 5 seconds, and 58°C for 15 seconds (supplement 1).

Criteria for SARS-CoV-2 variant definition

The presence of one or more mutant genes indicates a presumptive positive result. Therefore, each assay should be interpreted with caution and with consideration of circulating variants. Accordingly, the L542R was reported for Delta. The detection of N501Y, A570D, and HV69/70del indicated presumptive Alpha. Similarly, the presence of one or more of the K417N, E484K, and N501Y mutations indicated Beta presumptive positivity. HV69/70del, K417N, and/or N501Y indicated Omicron, whereas E484Q and L452R reported Kappa. K417T and/or E484K indicated Gamma and was assumed to be positive [6,7].

Statistical analysis

For a continuous variable, descriptive statistics were used. STATA statistical software (STATA version 16 StataCorp LLC., College Station, TX, USA) was used to compute the Ct values, relative frequency, and percentage. Variables with a categorical nature are shown as percentages.

Results

Socio-demographic characteristics and sample distribution

A total of 641 study samples were enrolled for RT-PCR testing. Of these, 374 were SARS-CoV-2–positive. All confirmed positive samples were screened for six S gene and Delta gene variant-typing RT-PCRs. Of the 374 PCR-positive study samples, 267 (71.3%) were positive for variant-typing RT-PCR, whereas the remaining 107 samples revealed negative results. Demographically, males were accounted for 57% of the total tested samples. The majority age group of the study participants was between 25 and 65 years (199 of 374, 74.5%) (Table 2).

The distribution of variants across the four waves based on the WHO classification

According to our study data, the Alpha variant was detected in waves I, II, and III in decreasing proportions and finally became undetectable in wave IV (Supplement 2). However, the Alpha variant was dominant in the first wave, with a prevalence of 15 of 30 (50%) and was detected along with other variants. Similarly, the Beta variant was detected in waves I-III but not in wave IV. The variant was dominant in the second wave, with a prevalence of 20 of 28 (71.4%).

In the third wave, the Delta was the dominant variant, with a frequency of 121 of 135 (89.6%) and a slight detection of other VOCs and VOIs. Delta has been detected since wave III and continued until the next wave. Omicron was the major variant during the fourth wave, with a frequency of 66 of 74 (89.1%). The Alpha and Beta variants were not detected during the Omicron period. The Kappa and Gamma variants were found at low frequencies. The Gamma variant was detected across all four waves, with a range of 2.9% to 10%. The Kappa variant was found with proportions of one (0.7%) and two (2.7%) in the third and fourth waves, respectively. As Figure 1 indicates, the Gamma variant was detected across all low-frequency waves.

The Figure 2 demonstrates the distribution of variants across the regions and Addis Ababa. The Omicron variant was not reported in Addis Ababa because samples were not collected from this site. Of the 267 RT-PCR–genotyped samples, 370 mutant genes were detected. The most prevalent mutant genes were L452R, 125 of 370 (34%), K417N, 93 of 370 (25%), HV67-70del, 67 of 370 (18%), N501Y, 38 of 370 (10%), K417T, 20 of 370 (5%), A570D, 15 of 370 (4%), and E484K, 8 of 369 (2%). The least frequent mutant gene was E484Q, with a frequency of 3 of 370 (1%) (supplement 3).

Table 2
Socio-demographic characteristics of the study participants across the four waves, Ethiopia Public Health Institute, 2023.

Characteristics	Number	Percent
Sex		
Female	113	42.3
Male	154	57.7
Age group		
5-14 years	4	1.5
15-24 years	42	15.8
25-65 years	199	74.5
>65 years	22	8.27
Residence		
Addis Ababa	185	69.29
Outside Addis Ababa	82	30.71
Total	267	100

Diagnostic RT-PCR Ct value vs variant-typing RT-PCR positivity

Our study showed Ct differences in the identified variants. A lower median Ct value was observed for Delta 20.1 (interquartile range 17.9-23) and Omicron 20.2 (interquartile range 18.2-22.3) variants, respectively. In contrast, higher Ct values were observed in the variants Alpha and Beta, 27.8 (22.8-34.4) and 27.6 (23.3-31) respectively, in a consecutive manner.

Variant identification was related to the low Ct of diagnostic PCR and likely determines the performance of RT-based variant detection. We categorized the study samples based on initial RT-PCR (BGI) Ct values as follows: <30, between 30 and 35, and >35.

The comparison of Ct values of the study sample is most related with RNA viral concentration in the NP samples (high, medium, and low viral loads). A significant difference was observed between group Ct values <30 and the remaining two groups' Ct values. The present study revealed that variants were identified in 243 of 267 (91%) of the study samples that have Cts <30, and in 16 of 267 (5.9%) of samples, the Ct value was between 30 and 35, assuming a moderate viral load. Eight (3%) of the variants detected had a Ct value >35. However, RT-PCR assays are mostly similar to NGS platforms that require a sample Ct value of <30.

When we see the comparative performance of the two variant detection assays, the six S gene mutant detection assay becomes more sensitive as the Ct values of the samples increase or the viral concentration of the samples decrease compared with the Delta screening assay (Figure 3).

Discussion

In our study, we used two variant-typing RT-PCR methods to detect relevant mutations and characterize the variants from confirmed repository-positive samples collected across different regions of Ethiopia. Our findings shed light on the genomic landscape of SARS-CoV-2 variants during the country's initial four consecutive COVID-19 epidemic waves.

Our study aligns with global observations, confirming that the Alpha, Beta, Delta, and Omicron variants were responsible for driving Ethiopia's first four waves of COVID-19 outbreaks. The finding is consistent with global SARS-CoV-2 variant reports and agrees with the study of Sisay *et al.* [8]. These variants have been closely monitored worldwide because of their potential impact on transmission dynamics and disease severity.

Besides the dominant variants, we also observed other variants with lower frequencies across all four waves. Our findings attributed the country's first SARS-CoV-2 wave to the Alpha variant. The variant was first identified in the United Kingdom in September 2020 and is responsible for increased infections in different parts of England. By the second

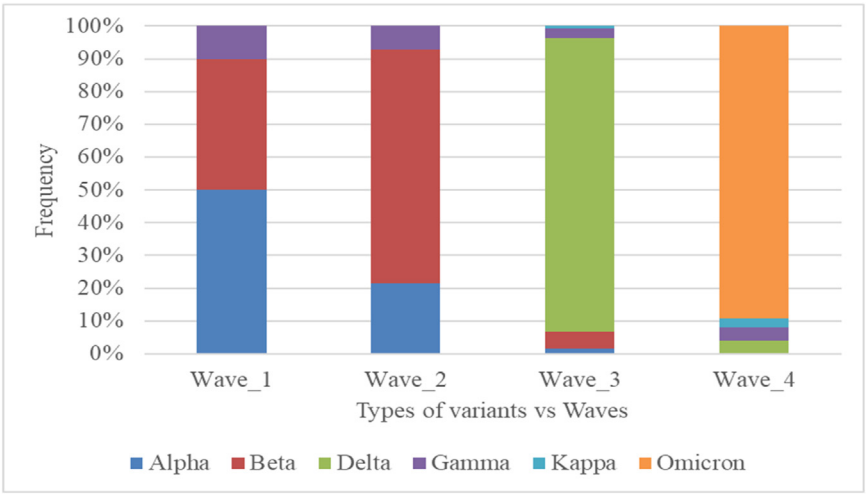

Figure 1. Distribution of variants among the four waves (N = 267, samples identified by variant-typing reverse transcription-polymerase chain reaction.

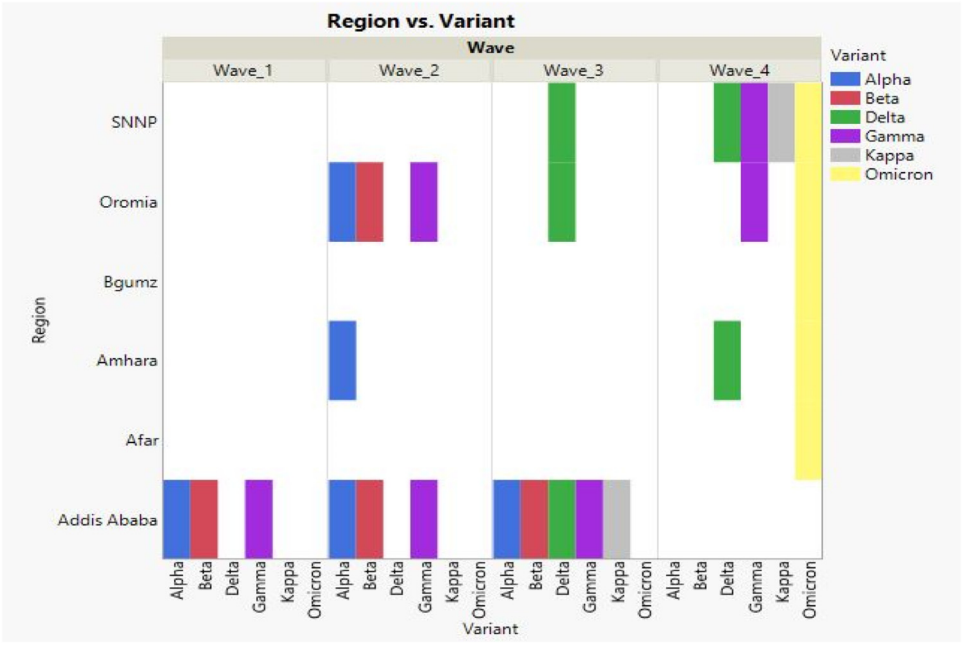

Figure 2. Distribution of SARS Co-V-2 variants across the four waves (n = 267), Ethiopia Public Health Institute, 2023.

quarter of 2021, the Alpha variant had accounted for most infections in the United States and many European countries [9]. The country's second wave was led by the Beta variant. This VOC was first detected in South Africa in December 2020. Between October 2020 and January 2021, South Africa's daily case report increased significantly from 2000 to more than 20,000 cases per day. This increment estimates that >30% of the population has already been infected. The receptor-binding domain (RBD) mutations K417N, E484K, and N501Y are associated with the Beta variant [10].

The findings of this study showed that the third wave of COVID-19 was mostly caused by the Delta variant. This variant was first identified in India in early 2021 and is highly contagious. It quickly spread to about 54 countries and rapidly replaced the Alpha variant in the United Kingdom and the United States. The Delta and Kappa variants share a common ancestor and have a mutation in the RBD; however, the Kappa variant has a different RBD mutation. According to our study, the fourth wave in the country was fueled by the Omicron variant. In the global arena, the first case of Omicron was confirmed in South Africa/Botswana in November 2021 [9].

The Gamma variant is also one of the significant VOCs among the identified variants of SARS -CoV-2. It was first identified in Brazil in early January 2021. This variant was responsible for the second wave in Brazil, which has a high infection rate and related mortality. The variant was transmitted from Brazil worldwide. The Kappa variant was also one of the VOIs identified in India. It was responsible for the second wave in India in April 2021. Of the mutations, L452R has a most notable mutation; E484K was also observed in a small frequency [11].

Several SARS-CoV-2 VOCs and VOIs share numerous key mutations. These mutations mostly existed in the S gene and RBD regions. The S protein enables attachment of the virus to the host cell surface angiotensin-converting enzyme 2 (ACE2) receptor, allowing SARS-CoV-2 entry into host cells [11]. The results of this study revealed the presence of key mutations in the regions of L452R, K417N, HV67-70del, K417T, E484K, N501Y, E484Q, and A570D, with varying frequencies. These key S mutations have an important impact on public health and allow variant characterization [9,10]. Of the identified mutations, 30 of them have been approved for investigation of VOC/VOI since the Alpha spike gene target failure has been recognized. L452R is the most predom-

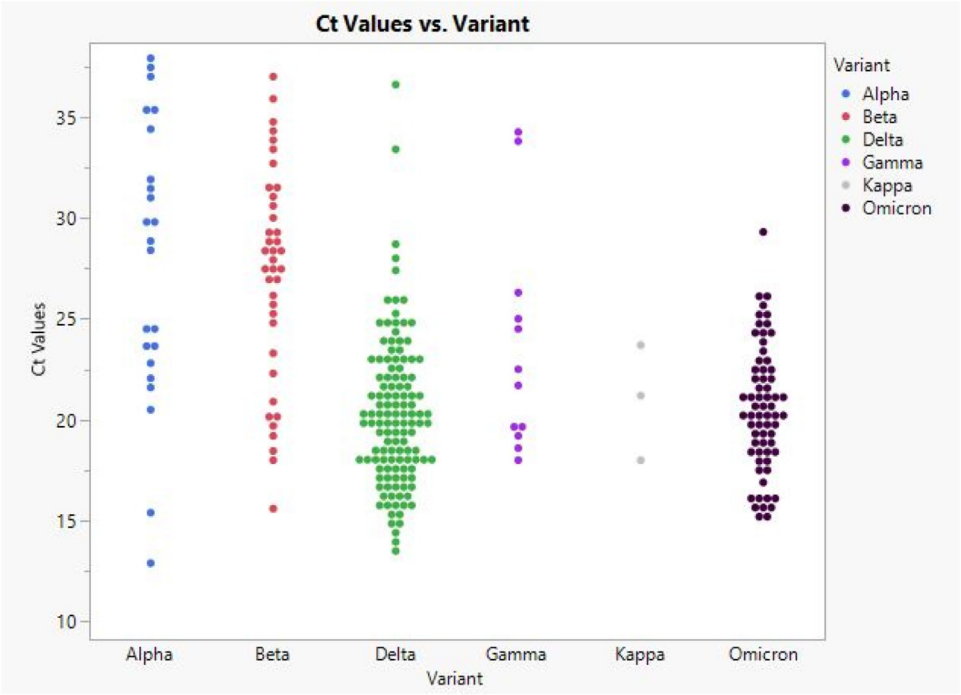

Figure 3. Variant-typing RT-PCR result distribution against Diagnostic RT-PCR Ct value category (n = 267).
Ct, cycle threshold; RT-PCR, reverse transcription-polymerase chain reaction.

inant S mutation observed in our study samples, with a frequency of 125 of 370 mutant genes. This mutation is associated with increased infectivity, higher transmission, and a reduction in neutralization by specific therapeutic antibodies. K417N is the second most predominant key mutation, with 93 of 370 (25%) present in the Beta variant as K417N and in the Gamma variant as K417T. K417N/T rarely occurs in the absence of other receptor-binding motif mutations, possibly because K417 mutations appear to reduce ACE2 binding and facilitate immune escape. The HV67-70del observation was 67 of 370; the deletion increases the binding affinity of the RBD of ACE2 and is also associated with S gene target failure in Alpha and Omicron. N501Y existed in 38 of 370. N501Y is commonly present in the Alpha, Beta, and Gamma VOCs and increases ACE2 affinity and virus replication in cells in the upper airway [12]. E484K was also detected, which is recognized by a high polyclonal antibody, developing within people infected with SARS-CoV-2. E484K is present in the Beta and Gamma VOCs and in the VOIs. E484K has been reported within several Alpha variant sub-lineages. The E484K mutation found within the RBD is a major target of neutralizing antibodies elicited during the primary exposure to the SARS-CoV-2 virus. In addition to the aforementioned key mutations, A570D and E484Q were observed in the study with lesser frequency.

Here, the Delta variant type by RT-PCR distinguished the presence of the L452R mutation in SARS-CoV-2–positive samples. The Delta PCR detected 81 of 83 (97.5%) from the NGS-confirmed Delta sample via the L452R probe. This variation could result from the number of study samples used (n = 28) and the target gene difference; our study kit targets the L452R gene for Delta identification. In addition, the difference may have resulted from a lower diagnostic RT-PCR Ct linked to a high viral load [12].

The SARS-CoV-2 qualitative RT-PCR test is sufficient for making a diagnosis. However, the Ct values may play a crucial role in predicting the accuracy of variant identification through RT-PCR. A low Ct of the diagnostic PCR is likely to determine the performance of RT-based variant detection. According to a recent study, 91% of positive samples (243 of 267) resulted in Ct values <30. The remaining 5.9% (16 of 267) of samples had a Ct between 30 and 35. Research conducted in South Africa has shown that it is difficult to genotype samples with high Ct values exceeding 35 using variant-typing RT-PCR. This outcome is different from the initial expectation [13].

Notably, 107 samples have tested negative with two variants of RT-PCR but positive with diagnostic RT-PCR. This negative outcome could be because of a high diagnostic PCR Ct value, a wild-type virus sample, or a mutation that cannot be detected by the tests used [12,13].

Our study finding showed that for the Delta and Omicron variants of the PCR target ORF1ab, the median Ct values were 20, whereas the Alpha and Beta variants were 27 cycles, which is significantly higher than the earlier two variants. Our finding contrasts with other studies, which could be owing to the possibly old samples recovered from the repository and the sample collection method [14].

PCR-based surveillance is an effective method of monitoring SARS-CoV-2. Although NGS offers a comprehensive variant detection, PCR-based surveillance is more cost-effective, scalable, and accurate. However, there are limitations to PCR-based surveillance, such as limited target coverage, difficulties in identifying variants across thousands of regions with single-base resolution, and being cumbersome for multiple targets [15]. RT-PCR genotyping is a useful tool when whole genome sequence (WGS) is unavailable or too expensive. It provides rapid results for patient management. In a recent study, RT-PCR results were 100% concordant with WGS for the Omicron and Delta variants in a blinded panel [16].

Our study data also showed that VOCs and VOIs were identified across the studied waves. According to the WHO classification, the Alpha, Beta, Gamma, Delta, and Omicron variants were identified; similarly, among VOIs, the Kappa variant was detected at a smaller frequency. Our finding is certainly concurrent with that of Sisay *et al.* [8].

It has been revealed that the limited capacity for WGS SARS-CoV-2 sequencing is hindering its scalability. Our study indicates that there are trends in variants of concern (VOCs) that are similar to those seen in other parts of the world. It has been observed that relying solely on WGS would overrepresent VOCs with highly correlated viral load concentration of the samples. Therefore, real-time RT-PCR genotyping is being considered as a practical solution for identifying SARS-CoV-2 variants. It has become pivotal to track SARS-CoV-2 variants to ensure a rapid public health response because the finding of this study is in concordance with other similar studies [16–20].

Using this finding can aid in our understanding of viral evolution and guide public health responses with shorter turnaround times for point-of-care testing.

Our esteemed reader should consider this while inferring because our study used a sub-sampling WGS and the diagnostic variant-typing RT-PCR–negative samples' final status was not confirmed by WGS.

Conclusion

In conclusion, variant-typing RT-PCRs are a highly promising method for identifying and tracking variants in resource-limited settings. It is important to note that the six S gene mutant detection assay is significantly more sensitive than the Delta screening assay, especially as the Ct values of the samples increase or the viral concentration of the samples decreases. Furthermore, the L452R mutation can be used as a marker to identify the Delta variant from identified S mutations. We strongly advise conducting a large-scale study with WGS before implementing these diagnostics in clinical management.

Declarations of competing interest

The authors have no competing interests to declare.

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Ethics and consent

The research proposal was approved by the Addis Ababa University College of Health Sciences, Medical Laboratory Sciences departmental research and review committee. Stored samples obtained during standard COVID-19 diagnosis are used in this investigation. The Ethiopian Public Health Institute granted permission for the study to be carried out.

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Author contributions

AdA and AtGe: conceptualization, designing, data collection, analysis, write-ups, and coordination. WG, KD, RD MB, AS: conceptualization, designing, data collection, and analysis. MaE, VC, and MaU read and commented on the manuscript. AdA and WG wrote the final manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The data that support the findings of this study are available from National Data Management Center of Ethiopian Public Health Institute. Data are, however, available from the authors upon reasonable request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijregi.2024.100363.

References

[1] World Health Organization *Genomic sequencing of SARS-CoV-2: a guide to implementation for maximum impact on public health*. Geneva: World Health Organization; 2021.
[2] World Health Organization Regional Office for Africa *Guidance on tools for detection and surveillance of SARS CoV-2 Variants Interim Guidance*, Geneva: World Health Organization; 2021. Version 1.0.
[3] European Center for Disease Control and Prevention *Changes to the list of SARS-CoV-2 variants of concern, variants of interest, and variants under monitoring*. Stockholm: European Centers for Disease Control and Prevention; 2022.
[4] Amhare AF, Tao Y, Li R, Zhang L. Early and subsequent epidemic characteristics of COVID-19 and their impact on the epidemic size in Ethiopia. *Front Public Health* 2022;**10**:834592. doi:10.3389/fpubh.2022.834592.
[5] Abera A, Belay H, Zewude A, Gidey B, Nega D, Dufera B, et al. Establishment of COVID-19 testing laboratory in resource-limited settings: challenges and prospects reported from Ethiopia. *Glob Health Action* 2020;**13**:1841963. doi:10.1080/16549716.2020.1841963.
[6] BGI PathoGenesis Pharmaceutical Technology Co. Ltd *Identification kit for B.1.617 Lineage (L452R and E484Q) of SARS-CoV-2*, Shenzhen, China: Wuhan National Bioindustry Base; 2020. Instruction for use.
[7] BGI PathoGenesis Pharmaceutical Technology Co. Ltd *Detection kit for 6 mutations in S gene of SARS-CoV-2*, Shenzhen, China: Wuhan National Bioindustry Base; 2020. Instruction Use.
[8] Sisay A, Tshiabuila D, van Wyk S, Tesfaye A, Mboowa G, Oyola SO, et al. Molecular epidemiology and diversity of SARS-CoV-2 in Ethiopia, 2020–2022. *Genes (Basel)* 2023;**14**:705. doi:10.3390/genes14030705.
[9] European Centre for Disease Prevention and Control/World Health Organization Regional Office for Europe *Methods for the detection and characterization of SARS-CoV-2 variants – first update*. Stockholm/Copenhagen: ECDC/WHO Regional Office for Europe; 2021.
[10] Tao K, Tzou PL, Nouhin J, Gupta RK, de Oliveira T, Kosakovsky Pond SL, et al. The biological and clinical significance of emerging SARS-CoV-2 variants. *Nat Rev Genet* 2021;**22**:757–73. doi:10.1038/s41576-021-00408-x.
[11] Chakraborty C, Sharma AR, Bhattacharya M, Agoramoorthy G, Lee SS. Evolution, mode of transmission, and mutational landscape of newly emerging sars-cov-2 variants. *mBio* 2021;**12**:e0114021. doi:10.1128/mBio.01140-21.
[12] Wang H, Jean S, Eltringham R, Madison J, Snyder P, Tu H, Jones DM, Leber AL. Mutation-specific SARS-CoV-2 PCR screen: rapid and accurate detection of variants of concern and the identification of a newly emerging variant with spike L452R mutation. *J Clin Microbiol* 2021;**19**:e0092621. doi:10.1128/JCM.00926-21.
[13] Umunnakwe CN, Makatini ZN, Maphanga M, Mdunyelwa A, Mlambo KM, Manyaka P, et al. Evaluation of a commercial SARS-CoV-2 multiplex PCR genotyping assay for variant identification in resource-scarce settings. *PLoS One* 2022;**17**:e0269071. doi:10.1371/journal.pone.0269071.
[14] Rabaan AA, Tirupathi R, Sule AA, Aldali J, Mutair AA, Alhumaid S, et al. Viral dynamics and real-time RT-PCR CT values correlation with disease severity in COVID-19. *Diagnostics (Basel)* 2021;**11** 1091. doi:10.3390/diagnostics11061091.
[15] Chaves-Blanco L, de Salazar A, Fuentes A, Viñuela L, Perez-Florido J, Dopazo J, et al. Evaluation of a combined detection of SARS-CoV-2 and its variants using real-time allele-specific PCR strategy: an advantage for clinical practice. *Epidemiol Infect* 2023;**151**:e201. doi:10.1017/S095026882300184X.
[16] Borillo GA, Kagan RM, Marlowe EM. Rapid and accurate identification of SARS-CoV-2 variants using real time PCR assays. *Front Cell Infect Microbiol* 2022;**12**:894613. doi:10.3389/fcimb.2022.894613.
[17] Marchini A, Petrillo M, Parrish A, Buttinger G, Tavazzi S, Querci M, et al. New RT-PCR assay for the detection of current and future SARS-CoV-2 variants. *Viruses* 2023;**15**:206. doi:10.3390/v15010206.
[18] Nasir A, Aamir UB, Kanji A, Bukhari AR, Ansar Z, Ghanchi NK, et al. Tracking SARS-CoV-2 variants through pandemic waves using RT-PCR testing in low-resource settings. *PLoS Glob Public Health* 2023;**3** e0001896. doi:10.1371/journal.pgph.0001896.
[19] Jiang W, Ji W, Zhang Y, Xie Y, Chen S, Jin Y, Duan G. An update on detection technologies for SARS-CoV-2 variants of concern. *Viruses* 2022;**14**:2324. doi:10.3390/v14112324.
[20] Dip SD, Sarkar SL, Setu MAA, Das PK, Pramanik MHA, Alam ASMRU, et al. Evaluation of RT-PCR assays for detection of SARS-CoV-2 variants of concern. *Sci Rep* 2023;**13**:2342. doi:10.1038/s41598-023-28275-y.