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SARS-CoV-2 variants typing using real-time reverse transcription-PCR-based assays in Addis Ababa, Ethiopia

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This is to certify that; the thesis prepared by Wodneh G/meskel entitled: “**SARS-CoV-2 variants typing using real-time reverse transcription-PCR-assays in Addis Ababa, Ethiopia”** and submitted in partial fulfillment of the requirements for a Master of Science degree in Clinical Laboratory Sciences (diagnostic and public health microbiology specialty) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abbreviations/Acronyms

ARMS	Allele refractory mutation system
COVID-19	Coronavirus Disease 2019 Caused by SARS-Cov-2
EPHI	Ethiopian public health institute
GISAD	Global initiative for sharing all influenza data
NP	Nasopharyngeal
NGS	Next generation sequencing
RT-PCR	Quantitative real time Polymerase Chain Reaction
RNA	Ribonucleic Acid
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
S-gene	Spike gene in SARS-CoV-2
SGTF	S gene target failure
SNP	Single nucleotide polymorphism
UK	United Kingdom
VOC	Variant of concern
VOI	Variant of interest
WGS	Whole genome sequence
WHO	World health organization

Abstract

Background: Severe Acute Respiratory Syndrome Coronavirus 2 is a single-stranded positive RNA that possesses 30,000 base pair in diverse variants. Even though genomic sequencing is a well-established variant detection method; but due to its cost and longer result turnaround time attributes less attractive for variant identification in resource-limited countries. These complexities pose the need for RT-PCR based variant typing.

Objective: This study aimed to determine the type of SARS-CoV-2 variants in the first four consecutive COVID-19 waves using variant typing PCR in Addis Ababa, Ethiopia.

Methods: A cross-sectional study was conducted from nasopharyngeal samples source from EPHI COVID-19 biobank repository. Samples were randomly selected from the first four waves based on their collection dates. A total of 641 NP samples were selected and tested for SARS Cov-2. RNA was extracted using Bioer NPA-32P instrument (Zhejiang, China) using the extraction kit from MagaBio Plus RNA Purification Kit II (Hangzhou, China). The SARS-CoV-2 detection carried out from 10 μl RNA and 20 μl RT-PCR fluorescent mix (Shenzhen, China). The Ct value less than 38 considered positive per the manufacturer. B.1.617 Lineage and 6 S-gene mutation (Shenzhen, China) PCR kits used for variant typing.

Result: From the 374 total tested for variant detection, 267 (71.4%) were identified by the variant typing kits. The remaining, 107(28.6%) were not classified by both variant detection kits. Alpha, Beta, Delta, and Omicron, were dominantly identified variants from wave one, two, three and four, respectively. Based on the WHO variant classification, Alpha variant was identified with high proportion from wave-1 but absent in wave-4. Similarly, Beta variant detected from wave 1 to 3 with varied proportion but dominant in wave-2. Delta variant entirely identified from wave 3 and 4; while dominantly identified in wave 3. Omicron was the dominant variant of wave 4. From the total identified positive study samples, 243/267 (91%) variants identified from samples had Ct values less than 30 in diagnostic RT-PCR. Besides to VOC; VOI also identified.

Conclusion: The study data demonstrated that RT-PCR type variants from confirmed SARSCoV-2-positive sample. RT-PCR based variant typing would provide additional screening opportunity; where sequencing opportunity is inaccessible. The assays could be implemented in laboratories that perform SARS-CoV-2 molecular testing.

Key words: SARS-CoV-2, whole genome sequence, variants, Ct value

1. INTRODUCTION

1.1 Background

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a single-stranded positive RNA that possesses 30,000 base pairs. Mainly, the genome encodes spike (S), envelope (E), membrane protein (M), and nucleoprotein of structural protein and the sixteen nonstructural proteins. The S gene encodes the spike protein that links the virus to a host cell and the host immune system recognized it as foreign. As a result, more mutations are accumulated in the spike gene than in the rest SARS-CoV-2 genome. SARS-CoV-2 like other RNA viruses prone to genetic change originates from erroneous replication during genetic recombination. Sometimes such process produce variants distinct from the wild types. Variants may occurs through spike gene mutation either by substitutions, deletion, insertion, or single nucleotide changes of the genome (1,2).

SARS-CoV-2, like other RNA viruses, is prone to genetic change result from a poor proofreading mechanism. The mutation of spike gene (S) of the virus gives variants that different characteristics from ancestral strains. These variants are usually associated with increased spread, virulence, or resistance to host immunity. By the middle of 2020, Alpha was the first variant reported from the UK. This variant typically shows weak amplification of the S gene target, termed S gene target failure (SGTF). Subsequently, different variants have been identified since the pandemic through genome analysis (3).

According to the virus's mutation features and public health impact mutation, WHO classify some SARS-CoV-2 variants into variants of concern and variants of interest. Variants of concern have increased transmissibility, severe disease conditions, increased hospitalizations or deaths; decrease vaccine efficiency. Alpha, Beta, Gamma, Delta, and Omicron are contained under the VOC class. Eta, Iota, Kappa, and Lambda are classified under variants of interest that are potentially less severe and monitored until meeting variant of concern criteria(3).

Recently, the WHO have categorized both groups under de-escalated Alpha, Omicron, Kappa, Gamma, Epsilon, Eta based on at least one of the criteria the variant is no longer circulating(3,4).

As of 18 May 2023, Ethiopia experiences five successive COVID-19 epidemic waves. The first wave was observed during the WHO Epidemic weeks 32 to 36 of 2020. The second wave was after a few months between WHO Epidemic weeks 9 to 17. The third wave was observed during Epidemic week 33 to 39 of 2021, the fourth was WHO Epidemic weeks 46 of 2021 to 3 of 2022 and the last fifth wave was in WHO epidemic weeks 20 to 28 of 2022(5,6).

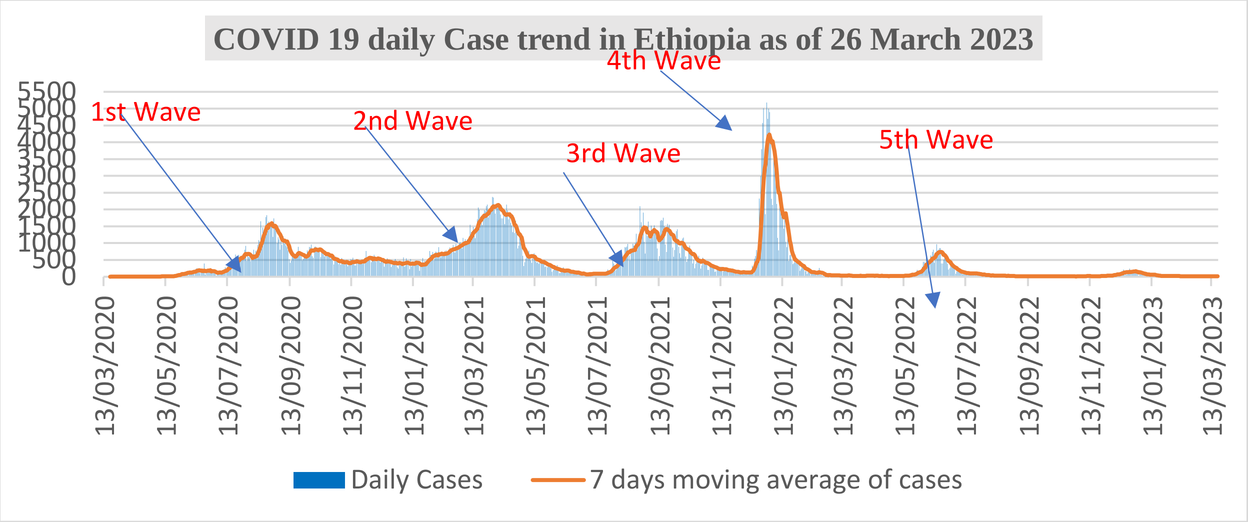

Figure 1 COVID-19 daily case trend in Ethiopia as of March 26,2023, EPHI (PHEOC COVID-19 Response)

The identification of SARS-CoV-2 variants requires widely available methods for detecting and tracking mutations. The whole genome sequence technology encompasses Ion Torrent, Illumina, and Oxford Nanopore platforms. However, in recent years PCR based variants detection using specific primers, probes or melt curve analyses have been introduced. The techniques are easy, rapid, and adaptable to existing PCR platforms(7,8).

The negative and positive SARS-CoV-2 RT-PCR test result is based on a definite cycle threshold value but in some instance the Ct value also serve as an indication of prognosis from certain treatment outcome. Besides this, the diagnostic RT-PCR cycle threshold determine PCR-based variants identification and sequence outcome(9).

This study objective was described variants that caused four successive national COVID-19 waves using two variants typing RT-PCR from positive samples. Furthermore, assessed the limits of the variant typing PCR against a range of diagnostic RT-PCR Ct values.

1.2 Statement of the problem

The rapid increase of the SARS-CoV-2 variants has necessitated a need to understand the nature of new variants to determine the public health measures.

Identification of variant is performed by the whole genome sequence through the next generation sequencing (NGS) technique. The technique limited to most advanced laboratories in high income countries. Besides this; it is labor-intensive, time-consuming, and difficult to perform at low cost. Therefore, variants types in many countries, like, Ethiopia not fully implemented, probably due to the lack of sequencing facilities. As a result, the variants that caused consecutive national COVID-19 waves not identified. In order to improve the timely detection of variants, WHO recommend all countries to perform genome sequence at least 1% of their positive cases. Due to the method resource intensiveness WHO for European region also recommend member countries to sequence much higher than 5% of their positive cases (10). This is partly due to methodological complexity and inherently NGS limited number of samples tested in single run. To overcome these limitation, alternative affordable RT- PCR-based variant typing technique has been introduced(7).

1.3 Significance of the study

As per our knowledge, this is the first study that addressed the first four COVID-19 waves; and could be initial reference for researchers who want to work in this area. As part of the pandemic mitigation, Ethiopia has established 72 NAAT-based COVID-19 test laboratories at every corner of the country from zero and have deployed trained personnel(11). But, very limited number of sequencing facilities that are more centralized in the capital in a centralized manner, which makes less likely it routine in Ethiopia. Thus; to overcome these limitations and to fill gaps, an affordable variant diagnostic method is needed. Since the occurrence of COVID; RT- PCR equipment availed in most regional laboratories; higher education owned referral hospitals and diagnostic institutions in Ethiopia. Therefore, RT-PCR based variant typing could be implemented in existing molecular laboratories reside in regions and epidemiological information gathering could facilitated.

On the other hand, Real-time PCR genotyping results are ready within 3 to 4 hours after the confirmed positive result; so that public health officials and clinician could use the presumptive variants' test result for appropriate public health intervention such as hot spot identification (7).

As of 18 May 2023, more than 15.5 million SARS-CoV-2 sequences were deposited in GISAID and publicly shared. Of these, Ethiopia accounts only 644 genome sequence; which more related with unavailability of enough number of advanced laboratories, supplies shortage, and insufficient bioinformatics capacity to interpret sequence data for timely action. Therefore, the variants that caused consecutive COVID-19 waves not fully identified (12).

Thus, this study has determined variants responsible for the first four consecutive national COVID-19 waves and the variant typing PCR level of detection has assessed against the diagnostic RT-PCR Ct value.

2. LITERATURE REVIEW

SARS-CoV-2 has had many genetic variations due to its higher mutation rates during replication. Viral recombination, a process by which genetic material is exchanged between viral sequences, also contributes to genetic changes. Over time, such changes can result the emergence of multiple variants that different from the wild type virus. Thus, variants may increase the virus transmission, leading to hospitalizations and increase mortality. Therefore, ongoing variants monitoring become essential (1,2).

Whole genome sequences a reliable specific variant characterizing method. It can be also a useful SARS-CoV-2 prevalence estimation tool, extracting variant level information through specialized bioinformatics. In other hand Whole genome sequencing an expensive, time-taking, and complex test procedure that can take from several days to generate results. These and other factors constraint applicability genome sequence in resource limited laboratories (1,13).Noticeably, there is a need for fast and simpler screening tool that can be used in a high-throughput fashion to increase the capacity of SARS- CoV-2 variant detection in real-time.

Since the occurrence of variants; several PCR-based typing methods have been developed and provide comparable result with whole-genome sequence(14). These methods mainly consist of the nucleic acid amplification detecting crucial spike gene mutations using the incorporated primer and probe(10). The RT-PCR workflow is cost-efficient as compared to most sequencing protocols and allow rapid screening of several specimens in short time (8). As a result, RT-PCR genotyping recommended by WHO and ECDC to determine different variants early detection from clinical samples.(5,11). The assays are widely used in the United Kingdom and France facilitate to discover the Alpha variants through targeting SGTF (16,17). The Roquebert B *et. al.* study the largest NAAT-based genotyping used two separate assays to screen HVdel69–70 and N501Y mutations from 123,867 samples has identified Alpha, Beta, Gamma and other several variants (16). Similar study conducted in Poland using RT- PCR targeting repetitive mutation sites able to determine epidemiological trends reflect the national COVID-19 waves(18).

Several methods have been developed for early detection of SARS-CoV-2 variants for diagnostic screening to generate preliminary results in a few hours. Many of these methods can identify the variants accurately, while others require subsequent confirmation by sequencing.

De Pace et *al*. evaluation of the diagnostic performance of five qualitative real time RT-PCR based tests as compared with NGS showed the overall accuracy of these assays ranged from 96.9% to 100% and 100% specificity and 96 –100%, sensitivity (19). Another study also evaluated the performance of RT-PCR assay target D950N gene of Delta variants showed 93% sensitivity and 100% specificity with NGS (21). However, all these assays only identify the presence/absence of specific mutations and do not allow to uniquely classifying all variants. Certainly, some mutations are common to different variants that may lead to an erroneous identification (20).

RT-PCR assays would help global variant surveillance of the SARS-COV-2 and fill the gaps about variants distribution and frequency. The Vogels CBF et *al*. indicate that multiplex RT-PCR assay can detect SARS-CoV-2 VOC and VOI from a variety of settings samples(21).Similarly the South Africa and Greek study reveal that PCR-based variant detection supports the variant surveillance activities(22,23).

The rapid variant typing methods restricted in identification of known variants. Hence ;continuous assay update with new targets is necessary (20).Moreover, the variant genome deviates from the primer target may give suboptimal results. Therefore, sometimes variant PCR recommended for circulating variants characterization (24). The most important causes that could lead to false negative result of RT-PCR based variant typing include thermal inactivation, storage time and temperature of specimen preservation, inadequate viral material; laboratory error; inappropriate specimen collection time and deficient sampling technique (25).

The cycle threshold at which fluorescent signals exceed from the background fluorescence signal and thus passes the threshold for positivity. Depending on the target gene RT- PCRs goes different cycle threshold to classify the sample negative or positive test result status. Sometimes RT- PCR measurement could categorize the viral load in to low, medium or high. In this case the NGS requires a sample has up to 30 cycle threshold of diagnostic RT-PCR for optimum gene coverage. Likewise, variants may less likely to be detected by RT-PCR because of low viral load in the clinical specimens(9). But study also show some variant PCR can detect variants from diagnostic PCR Ct value up to 35 cycles (23,26).

3. OBJECTIVES

3.1 General objective

To identify SARS-CoV-2 variants using real-time reverse transcription PCR that cause four successive national COVID-19 waves.

3.2 The Specific objectives

The specific objectives of this study were to;

- identify SARS-CoV-2 variants that caused four successive national COVID-19 waves,
- assess the ability of variant typing RT-PCR in ranges of Ct values determined by routine SARS CoV-2 PCR method, and
- identify variant of concern and interest

4. HYPOTHESIS

RT-PCR based SARS CoV-2 variant identification produce similar result to whole genome sequence based assays

5. METHOD AND MATERIALS

5.1 Study setting

The study was conducted at the Ethiopian public health institute, Parasitology COVID-19 Testing Laboratory (27). It is one of the COVID-19 testing laboratories, established during the pandemic in March 2020. As part of SARS CoV-2 surveillance and testing, samples are mainly referred from different sub-cities of Addis Ababa for suspected, community screening and patient follow-up of COVID-19. After diagnostic testing, all SARS-CoV-2 positive and 10% of negative samples were stored at -80^{o}C for sequencing purposes.

5.2 Study design and period

A cross-sectional study was conducted from August to October 2022.

5.3 Source Population

SARS-CoV-2 nasopharyngeal samples has been stored at EPHI for further surveillance purpose and diagnostic sample residual were used.

5.3.1 Study population

Study samples sorted according to their sample collection period represented as wave-1, 2, 3, and 4, and SARS-CoV-2 positive with BGI fluorescent RT-PCR have less than 38 cycles threshold. A total of 641 NP was selected from stored samples to address four COVID-19 waves. All wave samples were screened for 6 mutant S genes and Delta genes. Samples negative for both mutant genes were kept at -80oC for further analysis by whole genome sequencing. The first wave was depicted from August 13 to September 15, 2020; the second wave was from March to May 2021; the third wave represented by samples collected from mid of September to October 2021 and finally mid-December 2021 to the end of January 2022 depicted the fourth wave(6,9,20 and 28).

5.4 Inclusion and Exclusion Criteria

5.4.1 Inclusion criteria

All RT-PCR positive samples collected in specified wave period; samples with adequate volume, stored at -80^{0}C, sample with enough information and properly labeled.

5.4.2 Exclusion criteria

Sample collected outside the four wave period, unlabeled sample, SARS-CoV-2 negative sample and sample not stored under -80^{0}C.

5.5. Study variables

5.5.1 Dependent variables

Number and type of S-gene target failure, identified variants across the wave, diagnostic RT-PCR cycle threshold.

5.5.2 Independent variables

Residence, travel history, and mutation not targeted in both variant typing PCRs.

5.6. Sample size calculation and sampling method

5.6.1. Sample size

Table 5.6. Sample selected from respective waves

Respective waves	Total number of sample selected from respective wave	RT-PCR Negative	RT-PCR Positive
Wave-1	138	68	70
Wave-2	191	143	48
Wave-3	232	56	176
Wave-4	80	0	80
Total	641	267	374

5.6.2. Sampling Method

List of samples arranged in Excel sheet in respective wave period; selection has made using a simple random sampling method.

5.7. Measurement and Data collection

5.7.1. Data collection procedure

Socio-demographic information; samples collection date; diagnostic RT-PCR Ct value documented; type and the number of mutant gene identified from each study sample documented in the data collection forms.

5.7.2. Laboratory procedure

5.7.2.1. RNA extraction

RNA was extracted from all NP samples on Bioer NPA-32P instrument (Zhejiang, China) a high-throughput automated workstation. MagaBio plus RNA purification kit II (Hangzhou, China) used for extraction. All processes were undertaken per the manufacturer's instruction. 300 ul vortexes NP specimen were added to 96 well plates columns #1 and #7 well. Then, the plate placed into instrument. The machine agitating the plates every 10 seconds. Extraction completed at 9 minutes. Finally, the 70 ul RNA extract was stored at -80ºC for subsequent testing procedure.

5.7.2.2. RT-PCR detection

A fluorescent RT-PCR (Shenzhen, China) commercial kit was used for 641 samples to detect SARS-CoV-2. The kit targets the ORF1ab gene. Briefly, 10 µl RNA added to the respective well contains 20µl of master mix solution. The amplification reaction was executed in QuantStudio5 DX real-time PCR system (catalogue number A34322, Thermal Fisher Scientific). The cycle conditions were the following: 50 °C for 20 minutes and one cycle; 95 °C for 10 minutes and one cycle; and step-3, 95 °C for15 seconds and 60 °C for 30 seconds last for 40 cycles. All procedures were performed based on manufacturer instruction. A cycle threshold less than 38 was considered as positive SARS-CoV-2 and proceed to variant typing using RT-PCR.

5.7.2.3. SARS-CoV-2 variant typing and interpretation

SARS-CoV-2-positive samples screened for mutant genes by means of two variants typing kit (Identification Kit for B.1.617 Lineage and 6 S-gene mutations detection kit (Shenzhen, China).

5.7.2.4. Delta SARS-CoV-2 variant identification

Briefly, 10ul of RNA was added to a 20ul reaction mix (Shenzhen, China). The kit comprised positive and negative controls; reaction mixes A and B contain sequence-specific primers and fluorescent probes target L452R and E484Q genes. Testing was carried out in the conditions: 50 ^{0}C for 10 min, followed by 1 min at 95 ^{0}C, 45 cycles of 95 ^{0}C for 5 seconds, and 58 ^{0}C for 15 seconds on QuantStudio5 thermocycler (Thermo Fisher Scientific, Waltham, MA, USA) (29). The presence or absence of particular mutant genes determined by ΔCt Allele. The ΔCt Allele was calculated the difference between CtAllele and CtORF1ab. The difference equal or less than six indicated particular mutant gene positive result. Otherwise, define the negative result for the target mutant gene (29).

5.7.2.5. The six S-gene mutant detection

10 µl of RNA was added to the reaction mixture of 20 µl (Shenzhen, China). The kit comprises positive and blank controls; 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 A570D, HV69-70del, K417T, and E484K genes. 2 tests for each specimen in a single run. The assay was carried out in the conditions of 50 ^{0}C for 10 min, followed by 95 ^{0}C for 1 min,45 cycles of 95 ^{0}C for 5 seconds and 58 ^{0}C for 15 seconds on QuantStudio5 thermonuclear (Thermo Fisher Scientific, Waltham, MA, USA)(30)

Mutant gene result interpretation

Allele	Δ Ct value	Mutant gene result
ΔCt Allele	Ct Allele - Ct ORF1ab	≤6 indicate positive for mutation gene
		>6 indicate negative for mutant gene

5.7.2.6. Criteria for SARS-CoV-2 variants typing

The presence of one or more mutations indicate presumptive positive for a particular variant. The L542R the first criteria for Delta variant. The presence of N501Y mutations distinguish the Alpha, Beta, and Gamma variants from the Delta variant. The N501Y, A570D, HV69/70del indicates presumptive Alpha variant. Similarly, the presence of one or more mutations of K417N, E484K, and N501Y indicates the Beta presumptive positive. HV69/70del and K417N and/or N501Y indicated Omicron. While E484Q and L452R indicated Kappa. K417T and/or E484K indicate Gamma presumptive positive(19).

5.8. Data quality assurance

Laboratory procedures were performed per the standard operating procedure and manufacturer recommendations. Sample and data were collected by trained professionals. Reagents lot number, expiry date, and storage condition documented. Positive, no template, negative, and extraction controls were included during the RNA extraction and master-mixing step. Data completeness and consistency have been checked before data entry. Any result that deviates from the manufacturer's instruction was repeated.

5.9. Data analysis

Descriptive statistics for continuous variable Ct values frequency and percentage were calculated using STATA statistical software. Categorical variables were presented in n (%). A two-by-two table was used to perform characteristics of both variant PCRs assays.

5.10. Ethical consideration

Addis Ababa University College of Health Sciences, Medical Laboratory Sciences, departmental research and review committee has approved the study on 25/11/2022(Ref. No MLS/143/22).

5.11. Dissemination of the result

The result of this study is presented to Addis Ababa university school of medical laboratory for partial fulfillment of master of Science degree in diagnostic and public health microbiology. In addition, technical report submitted to Ethiopian public health institute. A manuscript is prepared for international journal submission. Furthermore, the result will be disseminated on national or international workshop or symposium.

6. RESULTS

6.1. Socio-demographic characteristics and sample distribution

A total of 641 study samples enrolled for RT-PCR test. Of these, 374 were found SARS-CoV-2 positive. All confirmed positive samples screened for 6 S-gene and delta genes continued by variant typing RT-PCRs. Among 374 diagnostic RT-PCR positive study samples, 267 (71.3%) became positive for variant typing RT-PCR while the remaining 107 samples revealed negative results. Further sampling of 176 wave-3 samples; 83 NGS-confirmed delta variant samples re-tested using Delta RT-PCR has showed strong agreement for Delta variant positivity 81/83(97.5%) in NGS. Demographically, males were dominant and accounted 57.7 %. The dominant age group of the study participants were between 25 to 65 was (199/374;74. 5%) as indicated in Table 1.

Table 1 **Socio-demographic characteristics of variant typing RT- PCR positive study participants in the four waves, EPHI, 2022.**

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

6.2 The distribution of variants based on WHO classification in the four waves

According to our study data, Alpha variant detected in wave 1, 2 and 3 with decreasing proportion and finally became undetected in wave-4. However; the Alpha variant was dominant in the first wave with a prevalence of 15/30(50%) and detected along with other variants. Similarly, Beta variant detected in wave 1 to 3 but not in wave 4. The variant was dominant in the second wave with a prevalence of 20/28 (71.4%).

In the third wave; Delta was the dominant variant with a frequency of 121/135 (89. 6%) with a slight detection of other VOC and VOI. The Delta detected since wave 3 and continue until the next wave. Omicron was the major variant during the fourth wave, with a frequency of 66/74 (89. 1%).Alpha and Beta variants were not detected during the Omicron wave period. Both Kappa and Gamma variants were found at low frequency. The Gamma variant has detected across the four wave with range of 2.9% to 10%. Kappa variant was found with proportion of 1(0.7%) and 2 (2.7%) in the third and fourth waves, respectively. As Table 2 has indicated the Gamma variant was detected across all waves with low frequency.

Table 2 **Distribution of variants in the four waves (n=267)**

Variant	**Wave-1 n(%)**	**Wave-2 n(%)**	**Wave-3 n(%)**	**Wave-4 n(%)**	**Total n(%)**
Alpha	15(50)	6(21.4)	2(1.5)	-	23(8.6)
Beta	12(40)	20(71.4)	7(5.2)	-	39(14)
Delta	-	-	121(89.6)	3(4.1)	124(46.4)
Gamma	3(10)	2(7.2)	4(2.9)	3(4.1)	12(4.5)
Kappa	-	-	1(0.7)	2(2.7)	3(1.1)
Omicron	-	-	-	66(89.1)	66(24.7)
Total	30	28	135	74	267(100)

Our study showed; cycle threshold differences among identified variants. The low median Ct value was observed from Delta variant 20.1[IQR 17.9-23] and Omicron variant 20.2[IQR 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] in a consecutive manner.

Among 267 RT-PCR genotyped samples 370 mutations identified using both variant RT-PCR. The most prevalent mutant gene was L452R 125/369(34%); K417N were 93/370(25%); HV67-70del were 67/370(18%); N501Y were 38/370 (10%); K417T were 20/369(5%); at last A570D were 15/370(4%) and E484K were 8/370(2%). The least frequent mutant gene was E484Q frequency of 3/370(1%).Table 3.

Table 3 ***Distribution of Spike mutations in the four waves samples (n=370), EPHI, 2022***

Spike mutation	Wave 1	Wave 2	Wave 3	Wave 4	Total (%)
E484Q	-	-	1	2	3(0.8)
L452R	-	-	122	4	126(33.7)
K417N	15	7	7	64	93(24.9%)
N501Y	15	17	3	3	38(10.2)
E484K	1	4	1	2	8(2.1)
HV69-70del	2	5	1	59	67(17.9)
K417T	3	7	3	7	20(5.4)
A570D	4	7	1	3	15(4)
TOTAL	40	47	139	144	370

Our study data revealed; despite to the number of study samples representing respective waves, the frequency and types of mutant gene detected in consecutive waves were increased. From 135 wave three study samples,139 mutant genes have been detected using variant RT-PCR. while from 74 wave four study samples; 144 mutant genes were detected. All target genes were identified in diverse proportion across the waves.

6.3. Diagnostic RT-PCR Ct value versus variant typing PCR positivity

Variants identification may have related to the low cycle threshold of diagnostic PCR and likely determines the performance of RT- based variant detection. We categorized study samples based on their initial RT-PCR Ct values as follows: less than 30, between 30 to 35, and greater than 35. This may have depicted high, medium, and low viral load.

The comparison of Ct values of the study sample with high, medium, and low viral loads is given in Table 4. A significant difference was observed between group Ct values less than 30 and the remaining two group Ct values. The present study revealed; 243/267(91%) of variants identified from samples that had cycle thresholds less than 30. While the remaining 16/267 (5.9%) of variants identified from samples Ct value between 30 and 35 assumed moderate viral load. The last category; 8(3%) of variant detected from Ct value greater than 35. However, the assay has sensitivity similar to NGS platforms that requires sample Ct value less than 30 for sequencing.

Table 4 ***Variant typing RT-PCR result distribution against SARS-CoV-2 detection Ct value (n=374)***

	Ct Value from routine SARS-CoV-2 ORF1ab test n(%)			
Both variant typing RT- PCRs result	**Less than 30**	**30-35**	**Above 35**	**Total**
Negative	51 (47.6)	32 (29.9)	24 (22.4)	107
Positive	243 (91.0)	16 (5.9)	8 (3.0)	267
Total				374
Delta gene kit result				
Negative	169 (68.4)	47 (19.03)	31 (12.5)	247
Positive	125 (98.4)	1 (0.79)	1 (0.79)	127
Total				374
Six S-gene mutant detection kit result				
Negative	176 (75.2)	33(14.1)	25(10.7)	234
Positive	118 (84.3)	15(10.7)	7(5.0)	140
Total				374

7. DISCUSSION

Our study showed; VOC/VOI can be screened by RT-PCR to facilitate epidemiological trends and identifying dominant variants that might reflect national COVID-19 waves. The Two real-time RT- PCRs have been used for relevant mutations and typing of variants from confirmed positive samples collected from April 2020 to January 2022 from different parts of the country. The study result explored Alpha, Beta, Delta, and Omicron were responsible variants for the country's the first four consecutive COVID-19 waves respectively. The finding consistent with global SARS-CoV-2 variants reports drive successive waves in different continents(31). Our result also agreed with the study of Sisay A.*et al*.(12). Besides dominant variants; another variant was also observed across all four waves. This could possibly be due to the narrow sampling period; that variant distribution could overlap with next-wave variants. Furthermore, Ethiopia is diplomatic hub for the African and center for many international organization connecting further transcontinental abroad. Hence the dissemination and diversification of variant was most likely a result of travel to and from Ethiopia via air and land transport(32).

Our finding described the country's first SARS-CoV-2 wave as resulting from Alpha variants. The variant was first identified in the UK in September 2020 and is responsible for the increased infection in different parts of England. By the second quarter of 2021, the Alpha variant accounted for the majority of infections in the USA and many European countries. In addition to HV69/70 deletions; E484K and N501Y mutations accumulated in Alpha variant (33).

Accordingly, the country's second wave was led by Beta variants. This Beta variant of concern was first identified in South Africa in December 2020. Afterwards, it is reported from Botswana between October 2020 and January 2021. The RBD mutations K417N, E484K, and N501Y and five NTD mutations are associated with the Beta variant (9,33 and 34).

This study data identified Delta variants was dominant in third wave of infections. The Delta variant was first identified in India in early 2021. The variant has increased transmission and spread to about 54 countries shortly and rapidly replace the Alpha variant in the UK and the USA. The variants share a common ancestor with Kappa variants containing the L452R mutation in the RBD, while the Kappa has the RBD mutation E484Q(4,24).

From our study; we have found that the country's fourth wave was initiated by Omicron dominant variants. In the global arena, the first case of Omicron was confirmed in South Africa in November

2021. Until officially report to the WHO, the South African COVID-19 epidemiological situation coincided with the Delta variants (33).

The gamma variant is also one of the significant VOCs that has worldwide distribution. It is first identified in Brazil from late 2020 to January. This variant is responsible for the second wave in Brazil. The variant has been transmitted out of Brazil and circulated throughout the world. Three significant mutations E484K, K417T, and N501Y have been documented (35).

There are 11 variants of interest under the category. According to the WHO as of 27 May 2021, the Kappa variant is one of the VOI identified in India. It was accountable for the second wave in India in April 2021.According to the WHO, the spike gene is the key region for monitoring mutations and shared by different VOC and VOI in common. These mutations may be related to the increased disease transmissibility, and chances of decreased therapeutic and vaccine efficacy. These mutations mostly exist in the spike gene and RBD region. The spike protein enables attachment of the virus to the human cell surface angiotensin-converting enzyme-2(ACE2) receptor to that facilitate SARS-CoV-2 entry to the human cell.

From a total of 370 mutant gene identified in this study; mostly identified from wave 3 and wave 4. Wave 3 represented by 135 study samples,139 mutant genes were detected along with all target genes in diverse proportion. While wave four depicted by 74 variant typing PCR positive samples, 144 mutant genes were detected. This may have associated with the characteristics of Omicron variants identified in the wave 4 possessed multiple mutant genes than any other variants(9).

From this study; we have identified key mutations L452R, K417N, HV67-70del, K417T, E484K, N501Y, E484Q and A570D in diverse frequency. These key spike mutations have an important impact on public health and allow variants characterization (3). L452R, the most prevalent spike mutation observed in our study samples in frequency of 125 out of 370 mutations. The L452R mutation primarily present and define Delta, Kappa and Epsilon variants(2,5). The mutations are associated with increased infectivity, higher transmission, and reduction in neutralizing therapeutic antibodies. The K417N, the second most prevalent key mutation, 93/370(25%); present in the Beta as K417N and in the Gamma as K417T. K417N/T rarely occurs in the absence of other RBM mutations, possibly because K417 mutations appear to reduce ACE2 binding and facilitate immune escape (28).

HV67-70del mutation has detected in proportion 67out of 370. The deletion increases the binding affinity of the receptor-binding domain of ACE2 and is also associated with S-gene target failure in Alpha and Omicron. N501Y mutation existed in 38 out of the total frequency of 369. N501Y is commonly present in the Alpha, Beta, and Gamma VOCs. It increases ACE2 affinity and virus replication in upper-airway cells (36). E484K was also detected; it is recognized by high polyclonal antibodies developing within persons infected with SARS-CoV-2. E484K is present in the Alpha, Beta, and Gamma VOCs and in the VOIs sub-lineages (37). The E484K mutation found in the RBD; a major target of neutralizing antibodies provoked during the primary exposure to SARS-CoV-2. Thus, this mutation is expected to affect antibody neutralization. Therefore, there is fear related to risk of reinfection due to the escaping from neutralizing antibodies. In addition to the aforementioned key mutations, A570D and E484Q were observed in this study in lesser frequency. Here, Delta variant typing RT-PCR detected the L452R mutation from confirmed SARS-CoV-2 positive samples. The Delta PCR detected 81/83 (97.5%) from NGS confirmed Delta sample. This slightly deviates from a similar study. The variation could resulted from the number of samples used(n=28) and the target gene difference; target the D950N gene(38); but our study kit targets the L452R gene for Delta variants characterization. In addition, the difference may result from genotyped sample diagnostic PCR cycle threshold was about 18.8 linked with high viral load(37).

A positive or negative qualitative SARS-CoV-2 RT-PCR test result that relies on cycle threshold may be sufficient for diagnosis; however, Ct values may be important to predict the identification of the accurate variants through RT-PCR. This study showed a low cycle threshold of diagnostic PCR likely determine the performance of RT-based variant detection. The present study revealed that 243/267 (91%) positive samples diagnostic RT-PCR Ct value was less than 30 while the remaining 16/267 (5.9%) samples Ct was between 30 and 35. This result is consistent with Wang. H *et al.* study that showed a mean diagnostic PCR Ct value range of 19.1 to 22.6 for the detection of diverse variants (39) and also agree with WHO recommendation (5,23). Thus, careful sample selection is essential for variant typing PCR procedure (41)

Notably, 107 samples have shown negative results by two variants typing RT-PCR. The analysis of a subset of SGTF negative samples diagnostic Ct data indicated negative outcomes were related to high diagnostic PCR Ct value. The sample would have the wild type virus or the applied assays may not identify some mutation (26). Another South African study revealed negative SGTF results linked to poor sample quality; mutations close to amino acid positions being analyzed, exist in primer binding sites that negatively impact, such as mutations at position HV69/70del (26).

The findings may exhibit that RT-PCR has better analytic sensitivity and work actually higher Ct value as compared with NGS, generally limited to samples cycle thresholds less than 30. However, some study claim variant PCR do not perform well in diagnostic cycle threshold greater than 35 cycles threshold(39). Currently, variant typing RT-PCR especially become important, because of lower viral loads as result of increased vaccination rate and also low viral load resulted due to various inherent sample related difficulties(14).

We tried to compare RT-PCR typed variants with their diagnostic RT-PCR Ct values considering as proxy of viral load to the identified variant. Our study data showed Delta and Omicron variants diagnostic RT-PCR Ct value median found 20; while the Alpha and Beta variants diagnostic RT-PCR median Ct were 27. Comparatively diagnostic RT-PCR cycle threshold of Alpha and Beta variants significantly higher than the previous two variants. The finding opposing similar study; showed the Alpha and Beta variants cycle threshold 21.8 and 22.1(16).This may be linked with the study sample. Our study sample recovered from the repository, and sample collection method. A literature review focused on RT-PCR Ct indicate a number of conditions affect Ct value. Specific to our situation may be disease stage at which the sample has been collected; primer targets and duration from sampling to analysis critical for Ct values (42).

Our study data also showed that variant of concern and interest identified. According to the WHO classification Alpha, Beta, Gamma, Delta and Omicron variants were identified; similarly, among variants of interest Kappa variant detected in smaller frequency. Our finding is certainly concurrent with Sisay A. *et al*(12). However, the 6-S gene detection method was designed to determine Alpha, Beta, and Gamma variants but the kit was able to detect the Omicron variants.

Limitations of the study

Due to small number of SARS-CoV-2 positive specimen availability in specific narrow period; waves may not have been represented with adequate number of study samples. The assays also focused only certain mutations (N501Y, A570D, HV69-70del, K417N, K417T, E484K, L452R, and E484Q) and the results are presumptive result. Hence, an additional confirmatory procedure required to know the actual variant type. Those 107 samples not categorize by variant typing PCR were not confirmed by sequencing to obtain actual method summary.

Conclusions

Our study indicates that two variant typing RT-PCR assays detect and differentiate most common variants Alpha, Beta, Delta, Omicron were associated in the first four successive national COVID-19 waves. Besides these, Gamma and Kappa variant also detected. Thus the assays could apply for diagnostic and surveillance.

In addition, the performance of Delta variant PCR proves acceptable agreement with NGS target the L452R mutation. The hospitals and diagnostic public health laboratories could use this assay as a means of SARS-CoV-2 variant diagnosis tests.

Recommendation

Variant RT-PCR assays could be feasible to use for variant determination in laboratory performing nucleic acid amplification test(NAAT). Therefore, we believe the assays facilitate prevention and control efforts toward future outbreaks associated with certain variants.

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9.ANNEX

Annex I: RNA extraction using nucleic acid purification system NPA-32P instrument

1. MagaBio plus RNA purification kit II brought room temperature
2. 1.5ul Eppendorf tube have labeled
3. The nasopharyngeal sample vortexed well
4. The 96 well plate kit has shaken
5. 300ul of sample added to 96 well plate column#1 and #7
6. 96-deep well plate has placed in to the instrument
7. Comb place correctly in to the position
8. Run the program press the save and run button
9. After the automatic purification the comb and 96 well plate
10. The extracted RNA has collected from 96 well plate column #5 and #11
11. 70ul of the product transferred in 1.5ul Eppendorf tube and stored -80 ^{0}C until the testing procedure

Annex II: Real-time Fluorescent RT-PCR Kit for SARS-CoV-2 Detection

Principles

RT-PCR combining fluorescent probing. Primers and sequence-specific fluorescence probes were designed to high conservative and specific region in SARS-CoV- 2, IFV A and IFV B genome. The probes were attached by fluorophores at the 5’ end as reporter with FAM for ORF1ab, with ROX for IFV B, and with CY5 for IFV A, and at 3’ end with quencher, respectively. In a meantime, specific primers and probes were developed using human housekeeping gene as internal reference with fluorophores VIC/HEX attached at 5’ end as reporter. RNA is reverse transcribed to cDNA. During the PCR procedures, the DNA polymerase cleaves the probe at the 5’ end and separates the reporter dye from the quencher dye when the probes hybridize to the target DNA. This cleavage results in the fluorescent signal generated by the cleaved reporter dye, which is monitored real-time by the PCR detection system. Measuring the fluorescence intensities during Real Time PCR allows the qualitative detection of SARS-CoV-2, IFV A and IFV B in specimens.

Reagent preparation

1. All reaction Mix thaw thoroughly at ambient temperature. Vortexed and centrifuge briefly. The enzyme mix kept on ice after thawed and vortexed.
2. The number of reactions (N) in the test, which includes Samples, Blank control (1 tube), Positive control (1 tube) has estimated and calculated.
3. 8-tube strips for PCR based on the estimated N of reactions has prepared and developed PCR mix as follow.

	SARS-CoV-2/IFV A/IFV B Reaction Buffer (µL)	**SARS-CoV-2/IFV A/IFV B Primers and Probes Mix (µL)**	**SARS-CoV-2/IFV A/IFV B B Enzyme Mix (µL)**
PCR-mix(ul)	15xN	3.5xN	1.5xN

Test procedure

1. 20µL PCR Mix per tube pipetted into 8-tube strips. Cap tightly and transfer to sample processing area.

2. 10µL RNA extract, Blank Control and Positive Control respectively added into the 8-tube strips prefilled with PCR Mix. Cap tightly and centrifuge briefly.
3. The fluorescent channels and reaction volume set 30ul
4. The sample put on QuantiStudio5 thermocycler. Amplification process proceeds as follow

Step	Cycle	Temperature	Time	Fluorescence measured(Y/N)
1	1 cycle	50°C	20 min	N
2	1 cycle	95°C	5 min	N
3	45 cycles	95°C	15 sec	N
		55°C	30 sec	Y

5. Quality control

Blank control: Ct values at FAM, CY5 and ROX channels no data available, Ct values at VIC/HEX channel are higher than 35 or no data available.

Positive control: Standard curves at FAM, ROX, CY5 and VIC/HEX channels are in S-shape with Ct values not higher than 35. otherwise the test is invalid

6. Result interpretation
    - Positive SARS-CoV-2, if standard curves at FAM, ROX or CY5 channel are in S-shape with Ct values less than 38, while standard curve at VIC/HEX channel is in S-shape with Ct value not higher than 35.
    - Negative SARS-CoV-2, if standard curves at FAM, ROX or CY5 channels are not in S-shape with Ct at FAM, ROX or CY5 no value or higher than 38.

Annex III. B.1.617 Lineage of SARS-CoV-2 (Fluorescence RT-PCR) identification kit

Principles

The mutation detection is based on allele refractory mutation system (ARMS)-based quantitative PCR (qPCR). For L452R, and E484Q of SARS-CoV-2, sequence-specific primers and fluorescent probes designed for variant. ARMS primers can specifically identify variant strain using different fluorescent bands, enabling to differentiate mutant strains (L452R and E484Q) from wild strain through 1 test for each specimen in a single run

Reagent preparation

1 Vortex and centrifuge briefly. Enzyme kept on ice.

2 The number of reactions (N) that includes Blank Control (1 tube), Positive Control (1 tube) estimated in order to prepare PCR-mix

3 PCR mix prepared for ORF1ab, internal reference, L452R and E484Q as follow

	Reaction mix(ul)	Enzyme mix(ul)
PCR- mix	18xN	2xN

Testing procedure

1 The kit vortexed and centrifuge briefly

2 20 µl of PCR-Mix added into respective wells of the strips.

3 10 µl of RNA added to prefilled strips PCR-Mix.

4 Finally, Positive control and Blank control added in PCR-mix.

5 The strip capped tightly and centrifuged at 2000 rpm for 10 seconds.

6 The sample put on QuantiStudio5 thermocycler. The amplification process proceeds as follow

Steps	**cycle**	**Temperature**	**Duration**	**Fluorescent signal collection**
1	1 cycle	50 ^{0}C	10 min	No
2	1 cycle	95 ^{0}C	1 min	No
3	45 cycle	95 ^{0}C	5 sec	No
		58 ^{0}C	15 sec	Yes

7 The presence of specific mutant gene calculated by ΔCt = CtAllele-CtORF1ab

8 The difference between Ct of specific allele and ORF1ab less than or equal to six it indicates the presence of specific mutant gene (positive result) otherwise it is negative

9 Type of variant defined by the presence of one or more variant defining mutant gene in the sample (as stated section 7.5.2.6)

Annex IV: Detection Kit for 6 mutations in S gene of SARS-CoV-2(ARMS-PCR)

Principles of the procedures

The mutation detection is based on allele refractory mutation system (ARMS)-based quantitative PCR. For N501Y, A570D, HV69-70del, K417N, K417T, and E484K of SARS-CoV-2, sequence-specific primers and fluorescent probes were designed for variant, respectively. ARMS primers can specifically identify variant strain using different fluorescent bands, enabling to differentiate mutant strains (N501Y, A570D, HV69-70del, K417N, K417T, and E484K) from wild strain through 2 tests for each specimen in a single run.

Reagent preparation

1 Vortex and centrifuge briefly. Enzyme kept on ice.

2 The number of reactions (N) that includes Blank Control (1 tube), Positive Control (1 tube) estimated in order to prepare PCR-mix. Prepared as followed

3 PCR mix prepared for ORF1ab, internal reference, internal reference, N501Y and K417N prepared as follow

	Reaction mix A (ul)	Enzyme mix(ul)
PCR- mix	18xN	2xN

4 Similarly, remaining gene PCR mix for E484K, A570D, K417TandHV69-70del prepared as follow

	Reaction mix B (ul)	Enzyme mix(ul)
PCR- mix	18xN	2xN

Testing procedure

1. Eight -tube strips of PCR has prepared based on estimated (N)number of samples.
2. Pipette 20 µl of PCR-Mix per tube of the 8-tube strips. Cover them and transfer to sample
3. 10µl RNA extract added into respective samples 8-tube strips prefilled with PCR-Mix.
4. 10µl Blank Control, and Positive Control respectively into 8-tube strips prefilled with PCR-mix
5. Cap them tightly and centrifuge them at 2000 rpm for 10 seconds.
6. The sample put on QuantiStudio5 thermocycler. The amplification process proceeds as follow

Steps	**cycle**	**Temperature**	**Duration**	**Fluorescent signal collection**
1	1 cycle	50 ^{0}C	10 min	No
2	1 cycle	95 ^{0}C	1 min	No
3	45 cycle	95 ^{0}C	5 sec	No
		58 ^{0}C	15 sec	Yes

7. The presence of specific mutant gene calculated by ΔCt = CtAllele-CtORF1ab
8. If the difference between Ct of specific allele and the ORF1ab is less than or equal to six it indicates the presence of specific mutant gene (positive result) otherwise it is negative

Annex V: Identification of all mutant genes determined by Δ Ct

Sample lab ID	Ct Mutant ORF1ab	Ct E484Q	ΔCt E484Q	P/N	Ct L452R	ΔCt L452R	P/N	Ct K417N	ΔCt K417N	P/N	Ct N501Y	ΔCt N501Y	P/N	Ct E484K	ΔCt E484K	P/N	Ct HV69-70del	ΔCt HV69-70del	P/N	Ct K417T	ΔCt K417T	P/N	CtA570D	ΔCt A570D	P/N

Annex VI: Sample selection and result tracking sheet

Wave No.__________

	SAMPLE HISTORY			SAMPLES TEST RESULT								SAMPLES DEMOGRAPHY				
				Delta genes		6 S-genes										
S.N	Samples Barcode reading	Lab ID	ORF1ab	E484Q	L452R	K417N	N501Y	E484K	HV6970del	K417T	A570D	Variant type	Sex	Age	Residence	Date of specimen collection

Annex VII: Declaration

Declaration

I, the undersigned agree to accept responsibility for the scientific ethical and technical conduct of the research project and for provision of required progress reports as per terms and conditions of the research publications office.

M.Sc. candidate: **Wodneh G/meskel(B.Sc.)**
Signature: ___________________
Date of submission: ___________________

This thesis has been submitted with our approval as advisors.

Advisor: **Regassa Diriba (Msc)**
Signature: _____________________
Date: _____________________
Place: Addis Ababa, Ethiopia

Advisor: **Kassu Desta (MSc, PhD candidate)**
Signature: ____________________
Date: ____________________
Place: Addis Ababa, Ethiopia

Advisor: **Abay Sisay (MSc, PhD candidate)**
Signature: ____________________
Date: ____________________
Place: Addis Ababa, Ethiopia

Advisor: **Adugna Abera (MSc, PhD candidate)**
Signature: ____________________
Date: ____________________
Place: Addis Ababa, Ethiopia

Advisor: **Atsbeha Gebreegiabxier (MSc)**
Signature: ____________________
Date: ____________________
Place: Addis Ababa, Ethiopia