S GENE TARGET FAILURE ANALYSIS IN COVID POSITIVE PATIENTS USING TAQPATH COVID-19 ASSAY

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This is to certify that **Ms. Sushma Acharya** has completed her thesis work entitled **"S GENE TARGET FAILURE ANALYSIS IN COVID POSITIVE PATIENTS USING TAQPATH COVID-19 ASSAY"** under my supervision.

This thesis work was performed to partially fulfill the Master of Science in Biotechnology award under the course code BT 621. The result presented here is her original findings. I, hereby, recommend this thesis for final evaluation.

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ACRONYMS AND ABBREVIATIONS

ACE2	Angiotensin Converting Enzyme 2
ARDS	Acute Respiratory Distress Syndrome
BALF	Bronchoalveolar Lavage Fluid
BSL-2	Biosafety Level 2
C	Cytosine
CDC	Centre for Disease Control and Prevention
CGICA	Colloidal Gold Immunochromatographic Assay
CoV	Coronavirus
CP	Conjugate Pad
CT	Cycle Threshold
DCs	Dendritic Cells
DIC	Disseminated Intravascular Coagulation
DPP4	Dipeptidyl peptidase 4
E	Envelope Protein
EDCD	Epidemiology and Disease Control Division
ER	Endoplasmic Reticulum
ERGIC	Endoplasmic Reticulum-Golgi Intermediate Compartment
G	Guanine
HCoV	Human Coronavirus
HDA	Helicase-Dependent Amplification
HE	Hemagglutinin Esterase
ICTV	International Committee for Taxonomy of Viruses
IFN	Interferon

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IRC	Institutional Review Committee
ISG	IFN-Stimulated Genes
kb	Kilobases
LAMP	Loop-mediated Isothermal Amplification
LFICS	Lateral Flow Immunochromatographic Strip
LRT	Lower Respiratory Tract
M	Membrane Protein
MDA	Multiple Displacement Amplification
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
MoHP	Ministry of Health and Population
N	Nucleocapsid Protein
NASBA	Nucleic Acid Sequence-Based Amplification
NC	Nitrocellulose Membrane
NPHL	National Public Health Laboratory
NPs	Nanoparticles
NSP	Non-Structural Protein
ORF	Open Reading Frame
PLpro	Papain-like Protease
PPE	Personal Protective Equipment
R0	Reproduction Number
RBD	Receptor Binding Domain
RCA	Rolling Circle Amplification

RdRp	RNA Dependent RNA Polymerase
RDT	Rapid Diagnostics Test
RNA	Ribonucleic acid
RPA	Recombinase Polymerase Amplification
Rpm	Revolutions per minute
RTC	Replication-Transcription Complex
RT-qPCR	Real-Time Reverse Transcription-Polymerase Chain Reaction
S	Spike Protein
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus-2
SGTF	S Gene Target Failure
SP	Sample Pad
TMPRSS2	Transmembrane Serine Protease 2
URT	Upper Respiratory Tract
UTR	Untranslated Region
VOC	Variant of Concern
VOI	Variant of Interest
VTM	Viral Transport Medium
WHO	World Health Organization

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ABSTRACT

The evolution of SARS-COV-2 resulted in the emergence of various variants of concern. A deletion at positions 69 and 70 of the spike glycoprotein precursor gene (S-gene), is associated with S gene Target Failure (SGTF) in a commonly used three gene diagnostic RT-qPCR assay and is one of the mutations that characterize the omicron and alpha variants till now. The use of TaqPath COVID-19 CE-IVD RT-PCR kit with a non-detectable S gene target and Ct value≤37 for either the ORF1ab or N gene targets was classified as SGTF. In this study, nasopharyngeal swab samples of covid positive patients visiting TU Biotech Corona Laboratory were used. Of the total positive samples, 24.26% (304/1,253) of the samples with low cycle threshold (Ct) values were selected to access SGTF. The rate of SGTF detection was high in the end of January which slowly decreased towards the start of next month. Among the 304 samples, 16.11% (49/304) samples resulted in SGTF whereas 83.88% (255/304) samples showed non-SGTF. Highest number of SGTF was found in the sample having the Ct values in the range 30-35 for both N gene and ORF1ab. Therefore, Ct mean value of SGTF samples were found to be greater than that of non- SGTF samples. Among the SGTF samples, 69.38% (34/49) of them were male and 30.61% (15/49) were female. The mean age of SGTF individuals was 38.20 years ranging from 8 to 73 years old. In the study population highest number of SGTF cases 29/49 (59.18%) belonged to 25-45 years age. Cough, fever and sore throat were the common symptoms in the SGTF patients. Among the SGTF samples the highest number of patients were vaccinated with Verocell followed by Covishield, Johnson and Pfizer vaccines. Detection of new variants of SARS-COV-2 with mutated spike protein through the SGTF approach helps in identifying circulating variants in the community.

Keywords: SARS-CoV-2, RT-qPCR, SGTF, non-SGTF, Ct value

CHAPTER 1

INTRODUCTION

1.1 Background of coronavirus

The term “coronavirus” refers to the spikes that resemble crowns on the virus’s outer surface (Shereen et al., 2020). Coronaviruses belong to the enveloped single-stranded ribonucleic acid viruses with a positive sense (+ssRNA) and non-segmented genome. They have the largest known RNA virus genomes (26.4-31.7 kb), with Guanine + Cytosine (G+C) contents ranging from 32% to 43%. Humans, mammals, and bird species can all contract coronaviruses. The coronaviruses are categorized into the Nidovirales orders, Coronaviridae family, and Coronavirinae subfamilies. The International Committee for Taxonomy of Viruses (ICTV) divides Human Coronaviruses (HCoVs) into four major genera. Alpha Coronaviruses (Alpha CoV), Beta Coronaviruses (Beta CoV), Gamma Coronaviruses (Gamma CoV), and Delta Coronaviruses (Delta CoV) (Mirzaei et al., 2020; Zawilska et al., 2021). Alpha and Beta Coronaviruses mostly infect mammals, while Gamma coronaviruses infect aves, and Delta coronaviruses infect both mammalian and avian species (Naqvi et al., 2020; Singh & Yi, 2021; Zawilska et al., 2021). Seven HCoVs have been identified so far: HCoVHKU1, HCoV-NL63, HCoV-OC43, HCoV-229E, Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV- 2). HCoV-229E and HCoV-NL63 are placed under Alpha CoV, whilst HCoV-HKU1, HCoV- OC43, SARS-CoV, MERS-CoV, and SARS-CoV-2 are classified as Beta CoV (Zawilska et al., 2021). They cause mild to moderate respiratory infections similar to the flu. But, MERS-CoV and SARS-CoV cause severe respiratory infections (S. Sharma & Bhatta, 2020).

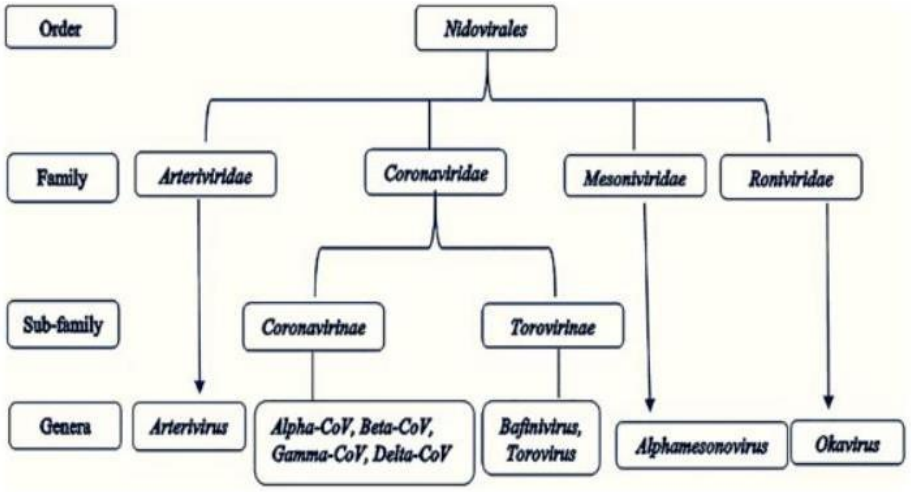

Figure 1: Taxonomy of Coronaviruses

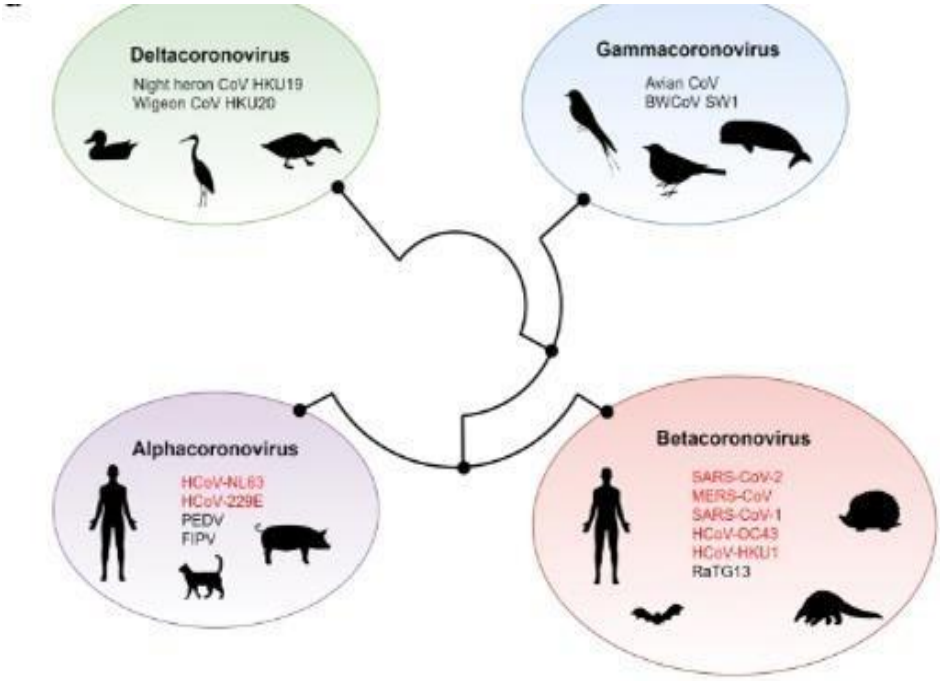

Figure 2: Classification of coronaviruses (Singh & Yi, 2021)

1.2 History of coronaviruses

In the middle of the 1960s, human coronaviruses were first discovered. SARS-CoV was the origin of the initial HCoV pandemic (S. Sharma & Bhatta, 2020). It began in Foshan, China's Guangdong Province, in November 2002 and lasted until 2014. It affected 28 nations and had 8096 cases and 774 fatalities, for a fatality rate of 10% (Microbiology & Chan, 2003). Patients who were infected showed signs of pneumonia and diffused alveolar injury, which

resulted in acute respiratory distress syndrome (ARDS). MERS-CoV, which started in Jeddah, Saudi Arabia, in June 2012, was the cause of the second HCoV pandemic (de Groot et al., 2013). There had been 2574 cases of MERS-CoV recorded in 27 countries as of the end of June 2021, with 886 deaths, for a case-fatality ratio of 34.4% (Zawilska et al., 2021). Mild upper respiratory injuries serve as the starting point for MERS-CoV infection, which progresses to cause severe respiratory disease. Similar to SARS-CoV, MERS-CoV patients suffer from pneumonia, ARDS, and renal failure (Memish et al., 2013).

1.3 About SARS-COV-2

A unique coronavirus outbreak with severe cases of pneumonia occurred in Wuhan, a developingcommercial hub of China, on December 31, 2019. Within the first fifty days of the epidemic, morethan 1800 people died and over 70,000 became sick. It was claimed that this virus belonged to the coronavirus family (Shereen et al., 2020). The novel virus was named n-Cov-19 or 2019-nCoV then the ICTV changed the name to SARS-CoV-2. Thus, the virus was named SARS-CoV-2, andthe disease COVID-19 or the 'Novel Coronavirus Disease 2019' on February 11, 2020, by the World Health Organization (WHO) (Almubaid & Al-Mubaid, 2021; Marahatta et al., 2020; Shereen et al., 2020).

With symptoms like dry cough, fever, exhaustion, headache, diarrhea, and sore throat, COVID- 19 is a highly transmittable and pathogenic infection that causes a respiratory illness (Peiris et al., 2004). In more severe cases, it may lead to pneumonia and difficulty breathing (Shereen et al., 2020; Zou et al., 2020). Furthermore, to acute respiratory distress syndrome and respiratory failure, COVID-19 is now recognized to present as systemic inflammation in high-risk patients, which can result in sepsis, acute cardiac damage, heart failure, and multi-organ dysfunction (Naqvi et al., 2020).

The majority of COVID-19 cases were initially identified only within Chinese borders and were frequently observed in travelers to the infected regions or in people who had close contact with such travelers. Due to the rapid international spread of these cases and the contagiousness of the COVID-19 virus, the WHO on January 30, 2020, designated the outbreak a Public Health Emergency of International Concern (S. Sharma & Bhatta, 2020). The WHO classified the outbreak as a pandemic on March 11, 2020 (Tsioutis et al., 2020). As of November 25, 2021, the WHO had received reports of 256,830,438 confirmed cases of COVID-19 worldwide, including 5,174,646 fatalities (WHO, 2021). Even though SARS-

CoV-2 is less lethal than SARS-CoV, it spreads significantly more readily (Petersen et al., 2020) and is highly challenging to identify, trace, and eliminate due to the long incubation period and nonexistent mild symptoms. Through its instructions, the WHO has urged the government to maintain medical facilities, increase public knowledge of the virus, and stockpile medical supplies since the disease's outbreak (K. Sharma etal., 2021).

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus is extremely contagious and is developing quickly. There are currently no viable treatments or controls, despite intense global efforts to prevent, manage, and contain the disease and break the transmission chain (Basnet, Pant, et al., 2021). Typically, the virus becomes more contagious and severe as a result of genetic modifications, including sequence variations (*Centers for Disease Control and Prevention*, n.d.). Therefore, to prepare for and stop future outbreaks, it is essential to understand the molecular origins and continuous evolution of SARS-CoV-2 (Singh & Yi, 2021). India has the most COVID-19 cases (101,139) among the South Asian nations, according to WHO research,followed by Pakistan (43,966), Bangladesh (23,879), Afghanistan (7,655), the Maldives (1,106), Sri Lanka (992), Nepal (402), and Bhutan (21) (Report & Asia, 2022).

Prevalence and case fatality ratio (CFR) of COVID-19 of top leading countries, neighbor countries of Nepal, and SAARC as of Jan 28, 2021.

Countries	Prevalence ratio on tested population (%)	Case fatality ratio (%)
TOP LEADING COUNTRIES		
USA	8.596	2.687
Brazil	31.681	2.721
Russia	3.767	2.170
NEIGHBOR COUNTRIES		
India*	0.099	1.460
China	0.055	5.297
SAARC		
Nepal	13.129	0.755
Pakistan	6.910	2.275
Bangladesh	14.769	1.661
Bhutan	0.188	0.128
Sri Lanka	3.710	0.542
Maldives	3.867	0.099
Afghanistan	22.281	4.799

Figure 3: Prevalence and case fatality ratio (CFR) of covid-19 (Basnet, Bishwakarma, et al., 2021)

Table 1: Comparative analysis of biological features of SARS-CoV-2, SARS-CoV, and MERS-CoV (Shereen et al., 2020)

Features	**SARS-CoV-2**	**SARS-CoV**	**MERS-CoV**
Emergence date	December 2019	November 2002	June 2012
Area of emergence	Wuhan, China	Guangdong, China	Jeddah, South Arabia
Date of fully controlled	Not controlled yet	July 2003	Not controlled (2,591 cases fromApril to August 2022) (WHO, 2022)
Key hosts	Bat	Bat, palm civets, and raccoon dogs	Camel
Number of countries infected	228	28	27
Entry receptor in humans	ACE2 receptor	ACE2 receptor	Dipeptidyl peptidase4 (DPP4)
Signs and symptoms	Cough, fever, and shortness of breath	Fever, malaise, myalgia, headache, diarrhea, shivering, cough, and shortnessof breath	Pneumonia, renal failure
Disease caused	SARS, COVID-19	SARS, ARDS	MERS
Total infected patients	631,656,122 (as of October 20, 2022)	8096	2574
Total recovered patients	610,532,744 (as of October 20, 2022)	7322	1688
Total died patients	6,577,612 (as of October 20, 2022; 1.04% mortality rate)	774(9.6% mortality rate)	886(34.4% mortality rate)

1.4 Source of spread

Animal CoVs are the source of zoonotic pathogenic viruses that infect humans (Ludwig & Zarbock, 2020). Numerous human activities involving close contact with wild mammals that are infected by the viruses offer numerous potentials for interspecies transmission. A coronavirus strain identified in a bat sample from Yunnan Province, China, in 2013 is one of the SARS-CoV- 2 strain's closest known relatives (D. Kim et al., 2020). The name of this strain is "RaTG13" indicating that it was found in a horseshoe bat, *Rhinolophus affinis*, in 2013. This strain's genomeshares 96% of its sequence similarity with SARS-CoV-2 (Singh &

Yi, 2021). Anti-SARS-CoV antibodies in Rhinolophus bats were also discovered, suggesting the bats as a potential source of viral replication (Shi & Hu, 2008). SARS-CoV-2 is 80% identical to SARS-CoV and only 50% similar to MERS-CoV (Nakagawa & Miyazawa, 2020). It's possible that SARS-CoV and MERS-CoV were passed from direct animal hosts, such as dromedary camels (*Camelus dromedarius*) for the latter and Himalayan palm civet cats (*Paguma larvata*) for the former, to humans. As the coronavirus identified from pangolins (*Manis javanica*) shares 99% nucleotide sequence homology with the virus that infects people, the researcher suggests that these animals may serve as a potential intermediate host for SARS-CoV-2 (Zawilska et al., 2021).

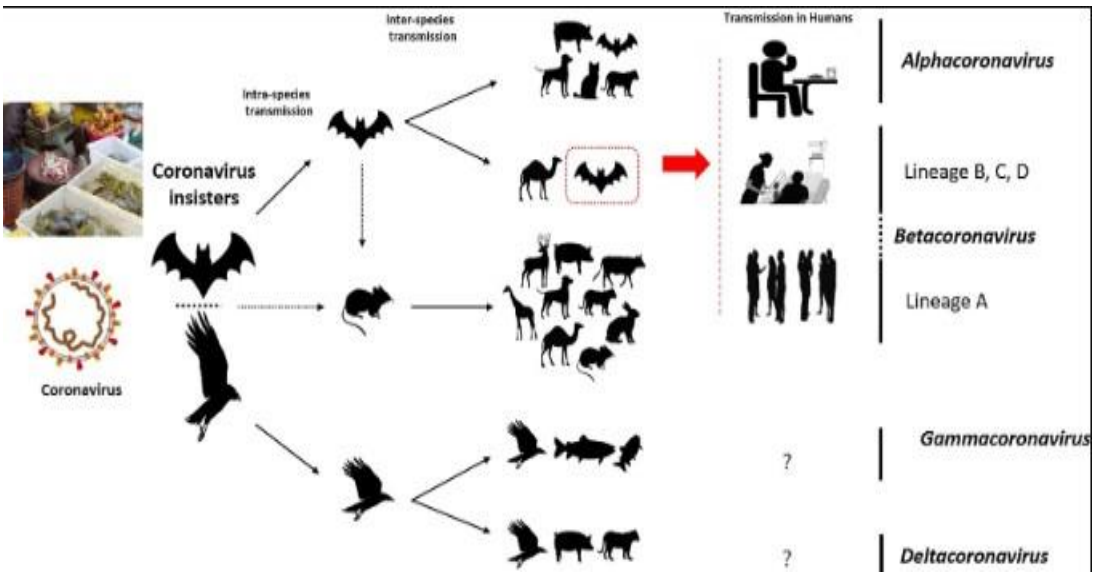

Figure 4: The key reservoirs and mode of transmission of coronaviruses (Shereen et al., 2020)

Since secondary and even tertiary transmissions between humans and other mammals are possible, widespread infection among humans raises risks to other mammals that interact with human populations. Such cross-specific transmission may facilitate the generation of recombinant SARS-CoV-2 strains that are potentially harmful. Therefore, it is crucial to eliminate within- and between-species transmissions through epidemiological, genomic, and functional analyses of variants (Singh & Yi, 2021).

1.5 Structure of Coronavirus

CoVs have the largest RNA viral genome, ranging from 26 to 32 kb in length (Lu et al., 2020). The linear, positive-sense, single-stranded RNA (+ssRNA) of the SARS-CoV-2 genome

has 29,891 nucleotides that can be translated into 9860 amino acids. The ribosomes of the host cells can directly translate the positive-strand genome's messenger RNA into viral proteins. The SARS- CoV-2 virus has a 3’ poly-A tail and a 5’ cap construct. A leader and untranslated region (UTR) sequence found at the 5’ ends of the genome contains the genetic material needed for RNA transcription and replication. The genome's 3' UTR region has 358 nucleotides, while the 5' UTRregion has 265 nucleotides (Lu et al., 2020). The SARS-CoV-2 genome organization is 5’-leader-UTR-replicase-S-E-M–N-3’ UTR-poly A with intrinsic genes inside the structural genes at the 3’ends of the genome (Fehr & Perlman, 2015; Liang et al., 2020).

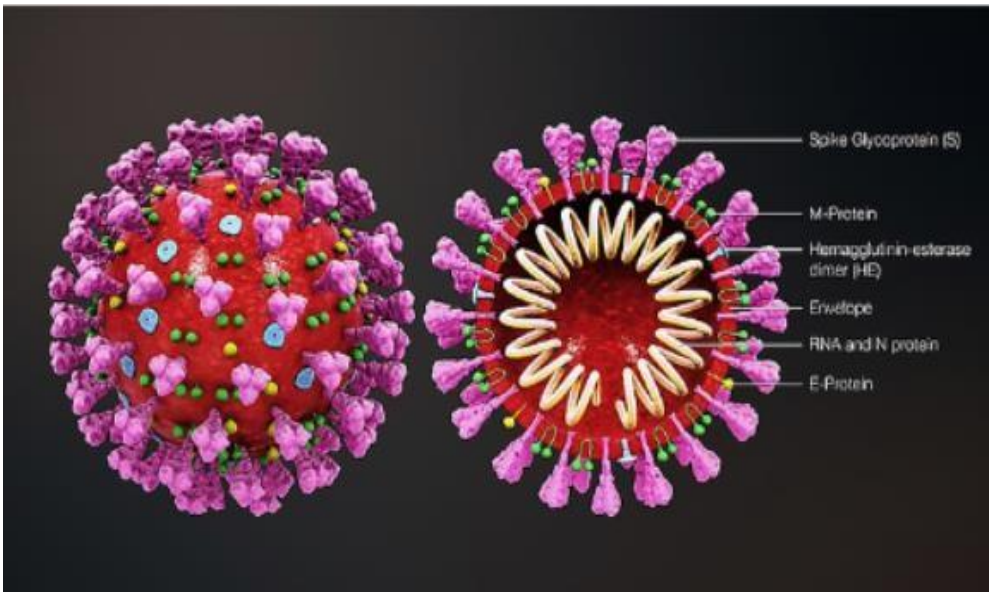

Figure 5: Coronavirus Structure (Sofi et al., 2020)

Genomic RNA of the SARS-CoV-2 has five caped and 3-polyadenylated segments, with seven Open Reading Frames (ORF). Two-thirds of the virus genome is made up of ORF 1a and 1b, which code for nonstructural proteins. NSP1 to NSP11 are encoded by gene ORF1a, and NSP12 to NSP16 by gene ORF1ab. The remaining ORFs encode structural proteins such as nucleocapsidprotein(N), spike protein(S), an envelope protein(E), and membrane protein (M) (Almubaid & Al-Mubaid, 2021; Mirzaei et al., 2020).

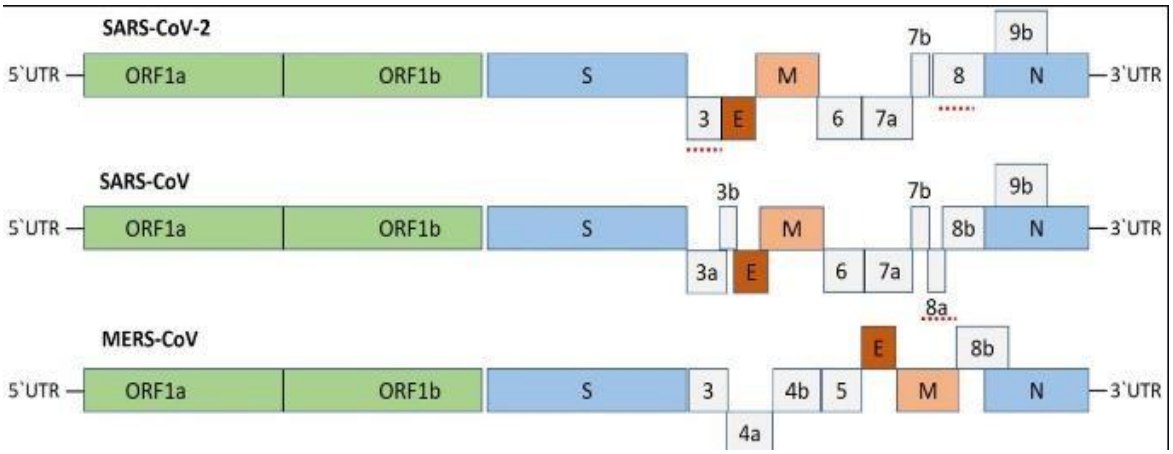

Figure 6: Beta coronaviruses genome organization. The dotted underlined in red is the proteinthat shows key variation between SARS-CoV-2 and SARS-CoV (Shereen et al., 2020)

Spike Protein

The S proteins are organized radially on the surface of the virus, giving the image from an electronmicroscope the appearance of a solar corona. There are 1273 amino acid residues in it. It has S1 and S2 as its two main subunits. The N-terminal galectin-like domain(S-NTD) and the receptor binding domain (RBD) are both present in the S1 subunit. Interacting with human ACE2 is essential for attachment to the host cell membrane (Hoffmann et al., 2020). Through its association with a transmembrane serine protease 2 (TMPRSS2) on the surface of the host cell, the S2 subunitaids in the viral fusion process. Specific viral surface epitopes are protected by the S protein glycosylation from being attacked by host antibodies (Arya et al., 2021).

Hemagglutinin esterase (HE) is another structural protein that exists in β-coronaviruses. It attaches to sialic acid on host glycoproteins and contains acetyl-esterase activity. It enhances the cell entrymediated to S protein and viral spread via the host mucus (Mirzaei et al., 2020).

Envelope Protein

The E protein is relatively small with 75 amino acids. It plays a significant role in viral morphogenesis and assembly (S. Li et al., 2020). It is a transmembrane structure that acts as viroporins forming protein-lipid pores in the host membrane which is involved in ion transport. By creating pores on the host membrane, the virion can escape from the infected cell. Its absence significantly reduces the viral load, and its presence is primarily

linked with the virulence of SARS-CoV-2 (Zawilska et al., 2021).

Membrane Protein

Membrane protein (M) is the main structural protein of the viral matrix and is composed of 222 amino acids. It consists of three domains (a short N-terminal ectodomain, a triple-spanning transmembrane domain, and a C-terminal endo domain). It plays a major role in RNA packaging(Tang et al., 2020). It also interacts with the S protein to trap the virus in the endoplasmic reticulum Golgi complex, where new virions are assembled and then released by secretory vesicles (Zawilska et al., 2021). It is involved in providing a distinct shape to the virus.

Nucleocapsid protein

Nucleocapsid proteins (N) play an important role in the packaging of viral RNA into ribonucleocapsid (Neuman & Buchmeier, 2016). N protein binds to the viral genome with a beads-on-a-string type pattern through its ~140 amino acid long RNA-binding domain. N protein has 419 aa. It mediates viral assembly by interacting with the viral genome and M protein, which are helpful in the augmentation of viral RNA transcription and replication (Naqvi et al., 2020).

1.6 Replication

The SARS-CoV-2 replication life cycle consists of five stages after infection: attachment, penetration, biosynthesis, maturation, and release. The initial step of infection involves the specific binding of the coronavirus S protein to the cellular entry receptor of the host. MERS-CoV recognizes the dipeptidyl peptidase 4 (DPP4) receptor. Whereas, SARS-CoV and SARS-CoV-2 recognize the angiotensin-converting enzyme 2 (ACE2) receptor of the host as a key receptor (Naqvi et al., 2020). In addition to receptor binding, for successful fusion, proteolytic cleavage ofthe S protein to S1 and S2 subunits by host cell-derived proteases, primarily transmembrane protease serine 2 (TMPRSS2), but also TMPRSS4, furin, cathepsin, trypsin, or a trypsin-like protease (HAT) of the human respiratory tract is required. The S protein binds to an extracellularpart of ACE2 through the receptor-binding domain (RBD) in the S1 subunit, followed by the fusion of the S2 subunit to the cell membrane. The envelope merges with the endosomalmembrane once it has entered the cell, releasing the viral genome into the cytoplasm where it is replicated and assembled

into new viral particles (Chambers et al., 2020; Costa et al., 2020).

The RNA (+) strand is first replicated to an RNA (-) strand, which is then employed for transcription of sub-genomic mRNA that can be translated into a variety of viral proteins or replication to another RNA (+) strand for new virion assembly. The translation of the replicase gene (encodes ORF1a and ORF1b) from the genomic RNA produces two polyproteins: replicase polyprotein 1a (pp1a) and polyprotein 1ab (pp1ab). Polyproteins pp1a compromise the eleven non-structure proteins (NSPs) including nsps 1 to nsps 11, while pp1ab compromises nsps 1 to nsps 16. The resulting polyproteins are cleaved by two cysteine proteases: papain-like protease (PLpro) and chymotrypsin-like protease (3LCpro; Mpro), located respectively within Nsp3 and Nsp5. Nsp1 plays a role in both translation inhibition and the degradation of the host mRNA.

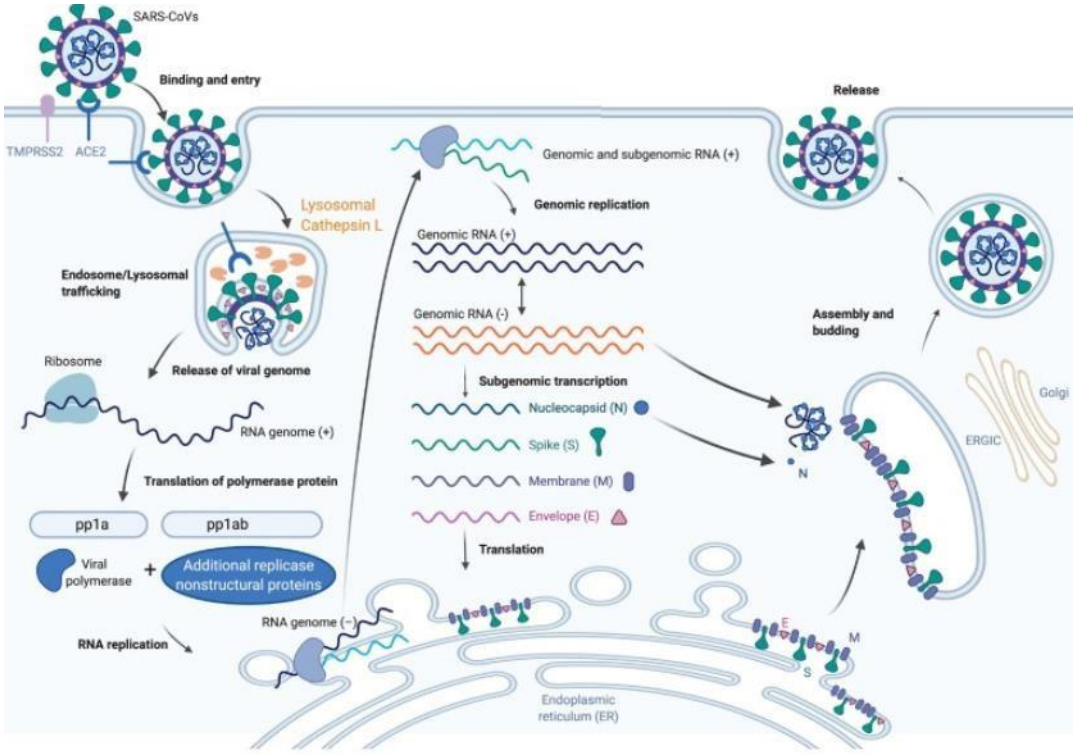

Figure 7: SARS-CoV-2 Replication cycle (Harrison et al., 2020)

The viral replication-transcription complex (RTC) is made up of Nsp2-16. The essential enzymaticactivities for RNA synthesis, RNA proofreading, and RNA modification are found in Nsp12–16, whereas Nsp2–11 is involved in intracellular membrane regulation, host immune evasion, and providing cofactors for replication. Nsp12, an RNA-dependent RNA polymerase (RdRP), and its two cofactors, Nsp7 and Nsp8, specifically carry out RNA

synthesis. Accessory proteins play a major role in modifying host defense mechanisms against infection and are regulators of viral pathogenicity (Mirzaei et al., 2020; Zawilska et al., 2021).

The translation of RNA encoding the N protein takes place in the cytoplasm, while mature formsof the M, E, and S proteins are formed in the rough endoplasmic reticulum (ER). The N protein surrounds the newly-synthesized RNA (+) strand, forming the nucleocapsid. Viral structures and nucleocapsids subsequently assemble in the Endoplasmic Reticulum-Golgi Intermediate Compartment (ERGIC). New virions packed in Golgi vesicles fuse with the plasma membrane and are released by exocytosis from the infected cell (Arya et al., 2021).

1.7 Transmission

Human CoVs are mainly spread by respiratory droplets, but during the SARS pandemic, reports of aerosol, direct contact with contaminated surfaces, and fecal-oral transmission were also reported (Harrison et al., 2020). SARS-CoV-2 also spreads primarily by respiratory droplets and, to a lesser extent, through aerosols produced during talking, coughing, or sneezing from symptomatic and asymptomatic individuals to others (Zawilska et al., 2021). The productive replication of SARS-CoV-2 in the upper respiratory tract (URT) and lower respiratory tract (LRT)supports direct transmission via respiratory droplets. Also, the rising number of cases demonstrates human-to-human transmission among close contacts who are coughing actively (Chan et al., 2020; Kujawski et al., 2020). The SARS-CoV-2 person-to-person transmission has an exponential growth rate because the primary reproduction number (R0) is approximately 2.6 (Lu et al., 2020). Some infected people may be contagious one to three days before the onset of the symptoms. Roughly 30% of people remain asymptomatic even though they become infected with SARS-CoV-2 (WHO, 2020).

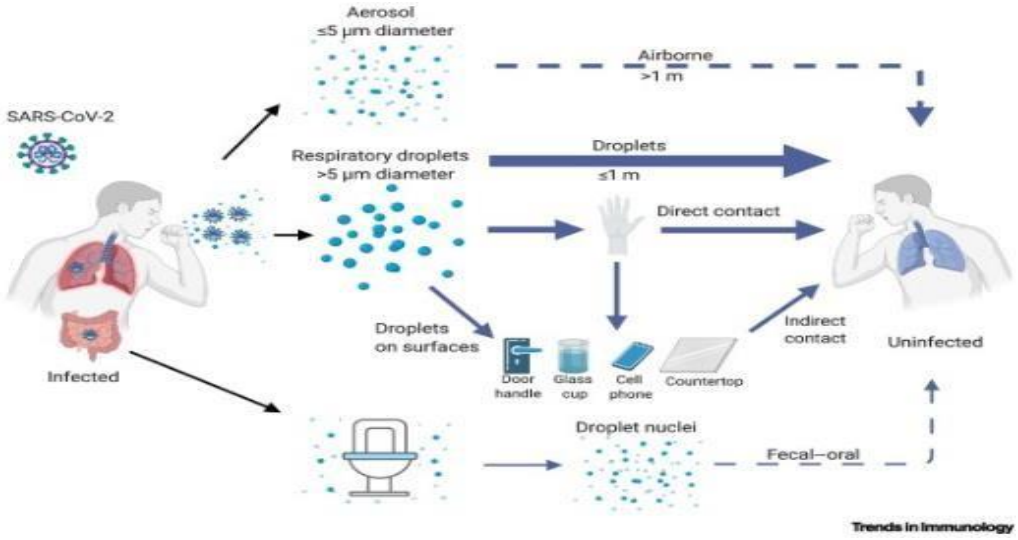

Figure 8: SARS-CoV-2 Transmission Routes (Harrison et al., 2020)

1.8 Pathogenesis

The incubation period, which is defined as the average amount of time between being exposed to the virus and the beginning of symptoms, typically lasts five to seven days but may extend to 14 days. The innate immune response is immediately activated when SARS-CoV-2 enters host cells. The virus must be able to suppress or avoid the host's innate immune signaling for successful infection (Harapan et al., 2020). Due to its efficiency and rapidity in removing viral infection, the IFN pathway is a prominent target of evasion. CoVs have a high sensitivity to IFN and block several IFN-stimulated genes (ISG) produced to inhibit viral replication (Totura & Baric, 2012). Patients with severe COVID-19 have an unbalanced immune response, with high levels of inflammatory cytokines and chemokines but low levels of circulating IFN-β or IFN-λ, leading to chronic viremia. Thus, evasion of IFN signaling by SARS-CoV-2 and impaired IFN production in human peripheral blood immune cells contribute the viral replication, transmission, and severe pathogenesis during COVID-19 (Harrison et al., 2020).

During the disease high levels of pro-inflammatory cytokines like IL-1, IL-2, IL-6, IL-8, IL-17, G-CSF, GM-CSF, and chemokines like IP-10 and MCP-1 are found in the sera and are crucial in the development of lung dysfunction by causing an overabundance of immune cells in the lungs. Increased in IL-6 levels increase the viral load and the attraction of inflammatory monocytes (Naqvi et al., 2020). In patients with acute COVID-19 (>55 years old), impaired T-cell activation from dendritic cells (DCs) and lower ability of CD4+ and CD8+ T-cells to

produce IFN- λ and IL-2 compromise an ideal adaptive immune response. Patients with severe COVID-19 symptoms have higher proportions of proinflammatory macrophages and neutrophils in the bronchoalveolar lavage fluid (BALF) compared to those with milder symptoms. The recruitment of neutrophils and monocytes may be driven by pulmonary endothelial cell dysfunction through vascular leakage, tissue edema, endotheliitis, and possibly, disseminated intravascular coagulation (DIC) pathways (Harrison et al., 2020).

1.9 Symptoms and Clinical Manifestations

Similar to SARS-CoV, the clinical signs of SARS-CoV-2 infection include fever, dry cough, dyspnea, chest discomfort, tiredness, and myalgia as the primary symptoms. Headache, dizziness, stomach pain, diarrhea, nausea, and vomiting are less frequent symptoms (Harapan et al., 2020). Elderly patients and those with comorbid conditions have more severe COVID-19 clinical symptoms, and a higher fatality rate. Hypertension, diabetes, cardiovascular disease, pulmonary disease, cancer, obesity, and chronic renal disease are the main comorbid conditions that might lead to severe COVID-19 (Zawilska et al., 2021). Some patients progressed to Acute Respiratory Stress Syndrome (ARDS) and septic shock, followed by multiple organ failure and even death. ACE 2 is abundantly present in ciliated cells of the airway epithelium and alveolar type II cells in humans, indicating the route of entry of the virus leading to ARDS and extensive lung damage (Harapan et al., 2020).

Although differences in immunological, genetic, endocrinological, social, and behavioral factors lead to men having a mortality rate that is 2.4 times higher than women, gender does not appear to affect susceptibility to SARS-CoV-2 infection (Jin et al., 2020). Due to immunologic and physiological changes during pregnancy, COVID-19 and other respiratory infections may be morecontagious in pregnant women (Mirzaei et al., 2020).

Table 1. Categories of COVID-19 severity according to the United States National Institute of Health (30).

NIH category	Signs and symptoms
Asymptomatic or presymptomatic infection	• Positive test for SARS-CoV-2 • No symptoms consistent with COVID-19
Mild illness	• Signs and symptoms of COVID-19 (e.g. fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhea, anosmia, ageusia) • No shortness of breath, dyspnea, or abnormal chest imaging
Moderate illness	• Evidence of lower respiratory tract disease during clinical assessment or imaging • Oxygen saturation (SpO_2) ≥ 94% on room air
Severe illness	• SpO_2 < 94% on room air • Ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2) < 300 mm Hg • Respiratory frequency > 30 breaths/min, or lung infiltrates > 50%
Critical illness	• Respiratory failure, septic shock, and/or multiple organ dysfunction

Figure 9: Covid-19 symptoms based on the category of illness (Zawilska et al., 2021)

1.10 Diagnosis

To direct epidemiological measures, infection management, antiviral therapy, and vaccine development, prompt detection and diagnosis are essential (Cui & Zhou, 2020). HCoV infectionshave experienced three unprecedented epidemics at the start of the twenty-first century, which have brought attention to the need for quick, accurate, and widely accessible diagnostic testing procedures (Loeffelholz & Tang, 2020).

1.10.1 Real-time reverse transcription-polymerase chain reaction (RT-qPCR)

The current gold standard for the diagnosis of COVID-19 involves the detection of viral RNA in the secretions from the respiratory tract of infected patients using a real-time reverse transcription-polymerase chain reaction (RT-qPCR) test (Chang et al., 2020). RT-qPCR assay targets the abundantly expressed genes such as the structural spike glycoprotein gene (S gene), envelop protein gene (E genes), nucleocapsid protein gene (N genes), the non-structural RNA-dependent RNA polymerase (RdRp), and replicase open reading frame 1a/b (ORF1a/b) genes. Designed primers targeting these genes are specific and sensitive for the novel SARS-CoV-2 and ruled outmost of the other coronaviruses (MERS, OC43, and 229E) and influenza viruses (H1N1, H3N2, H5N1, etc.) (Cui & Zhou, 2020).

Sample collection, sample preparation, nucleic acid extraction, reverse transcription, and PCR with real-time fluorescence signal detection are necessary for SARS-CoV-2 identification utilizing RT-PCR. RT-PCR has a high specificity and sensitivity for detecting viral nucleic acids. Similar to PCR, but using real-time monitoring of the fluorescence signal to assess the amplified nucleic acid, RT-PCR generates cycle threshold (Ct) values that are inversely proportional to the concentrations of the target nucleic acid in samples (Lim & Lee, 2020).

Target gene Ct values below and above the cut-off values, respectively, indicate positive and negative for the gene. To evaluate the effectiveness of the procedure and the presence of impurities, positive and negative controls are included in each reaction. Low viral loads in specimens can cause Ct values to be close to the cut-off, which can lead to false-negative or false- positive results. In account of the patient's history and clinical symptoms, it is important to carefully interpret the results of RT-PCR (Lim & Lee, 2020). False-negative results could also come from mutations in the viral genome, the presence of PCR inhibitors, the use of antiviral medication beforehand, and poor specimen quality. Additionally, the majority of RT-PCR tests take 4 to 6 hours to complete from the preparation of the specimen to the RT-PCR. Hence, additional advancements are needed, such as a shorter turnaround time for testing in the ongoing COVID-19 epidemic (Y. J. Kim et al., 2020).

1.10.2 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP)-based methods were created to get beyond RT- qPCR time and money-consuming limitations while still being able to detect infections' nucleic acids. It uses a DNA polymerase with chain displacement activity, 4-6 specifically created primers, and a steady temperature of 60–65 °C to deliver nucleic acid amplification in a short amount of time. The rolling circle amplification (RCA), nucleic acid sequence-based amplification (NASBA), recombinase polymerase amplification (RPA), multiple displacement amplification (MDA), and helicase-dependent amplification (HDA) are other isothermal amplification techniques in addition to LAMP (Cui & Zhou, 2020).

1.10.3 Lateral flow immunochromatographic strip (LFICS)

The lateral flow immunochromatographic strip (LFICS) developed and approved in China

for diagnosis of COVID-19 is portable, inexpensive, and doesn't require power. It functions like a pregnancy test and includes a sample pad (SP), conjugate pad (CP), nitrocellulose membrane (NC), and absorbent pad. IgM and IgG antibodies against the SARS-CoV-2 virus can be found in human blood within 15 minutes using the colloidal gold immunochromatographic assay (CGICA), which is based on gold nanoparticles (Au NPs) (Cui & Zhou, 2020). According to (L. Li et al., 2020) study, the testing sensitivity and specificity of the LFICS assay are 88.66% and 90.63%, respectively. Comparatively, the diagnostic sensitivity of an RNA-only test is 67.1%; when total antibody and RNA detection are combined, the sensitivity is 99.4% (Zhao et al., 2020).

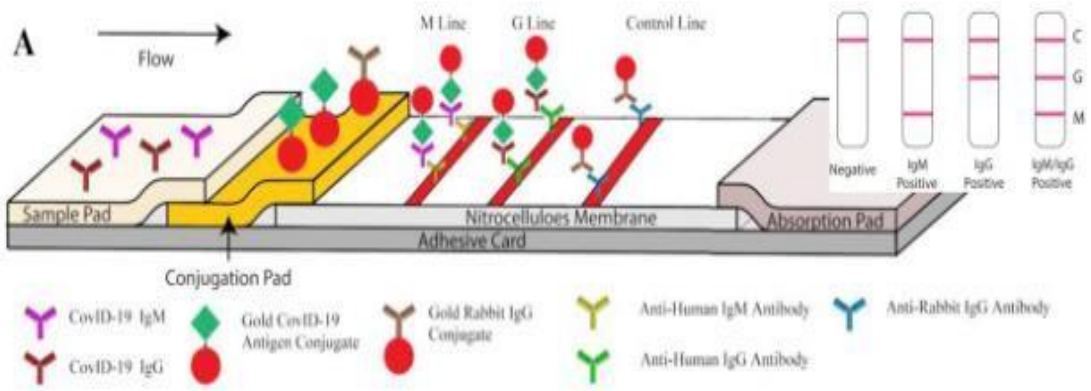

Figure 10: Schematic diagram of the LFICS based on Au NPs colloid for COVID-19 IgM/IgG antibodies detection (Cui & Zhou, 2020)

1.10.4 Antibody detection using serological tests

Infected people might not test positive for viral RNA, especially in the early stages of the illness, but it can be determined in retrospect that they had an immune response (Loeffelholz & Tang, 2020). During viral infections, immunoglobulin M (IgM) serves as the initial line of defense. Immunological memory and long-term immunity are both mediated by high-affinity immunoglobulin G (IgG) (Cui & Zhou, 2020). IgM and IgA antibodies are detected in 3 to 6 days(median 5 days), whereas IgG antibodies are detected 10 to 18 days (median 14 days) after symptom development. Therefore, early COVID-19 instances cannot be screened using detection technology that targets antibodies. For cases in the convalescent period, it is more beneficial. Serological testing can undoubtedly help identify asymptomatic COVID-19 patients who may spread the virus. It can also be utilized to

diagnose COVID-19 in the late stages, even in viral RNA-negative patients (Lim & Lee, 2020). Additionally, the correlation between viral neutralization titer and antibody detection levels is crucial for the development of vaccines. The sensitivity and specificity of diagnosis can be greatly increased by combining antibody detection techniques with quantitative real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) (Cui & Zhou, 2020).

1.10.5 Antigen detection

Rapid antigen tests (RDTs) detect the presence of viral proteins (antigens) in respiratory specimens. The RT-PCR-based diagnostic tests that are currently available are not reliable because they are still missing several infected cases. Additionally, they are not readily accessible in distantareas and developing nations that lack well-equipped laboratories and qualified specialists (Lim & Lee, 2020). Rapid antigen assays would have the benefit of quick turnaround times and affordable HCoV detection, but they are likely to have low sensitivity. They may have increased diagnostic sensitivity if specimens are collected while viral titers are at their peak (Loeffelholz &Tang, 2020).

1.11 Current Scenario of Covid-19

As of October 20, 2022, the total number of people infected with coronavirus worldwide is 631,656,122. 14,545,766 is the total number of active cases among which 14,507,192 (99.7%) are in mild conditions and 38,574 (0.3%) are critically ill. Whereas, among 617,110,356 closed cases 610,532,744 (99%) have already recovered or discharged with 6,577,612 (1%) fatalities (Worldometer,2022). 484,358 new cases have been identified worldwide within the last 24 hours according to the WHO Coronavirus (COVID-19) Dashboard (WHO,2022).

In Nepal, 1,000,325 cases have been recorded as of October 20, 2022, among which 987,580 (98.7%) cases have already recovered whereas 12,018 (1.2%) people have died due to covid infection. 50 new cases along with 51 cases of recovery from 727 active cases have been recordedwithin the last 24 hours (Ministry of Health and Population, 2022).

1.12 Rationale

The SARS-COV-2 pandemic highlighted the necessity of molecular surveillance at local, state, and national levels for identifying, monitoring, and containing the spread of the

virus. RT-qPCR technique will help in separating the highly contagious VOCs circulating in the community. The TaqPath COVID-19 PCR kit is used to identify the S gene target failure (SGTF) in infections caused by the dominant omicron during the beginning of the third wave in Nepal, demonstrating the value and significance of molecular assays. S gene dropout stands as a reliable marker for omicron variant identification, pending confirmation by sequencing. This method allows for the faster detection of this variant compared to the variant that caused past infection spikes indicating a growth advantage for this mutant. Therefore, being able to distinguish between the more transmissible and deadly SARS-COV-2 pathogens on time will help in gathering preliminary data on the appearance and spread of the virus. The findings of this research might offer a preliminary understanding of developments in the ongoing evolution of SARS-CoV-2. The reporting of early instances and clusters can help us better understand the epidemiology of COVID-19 now and in the future, as well as guide public health and social policy.

1.13 Statement of the problem

The highly contagious severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) quickly overran both the private and public healthcare systems globally. To track the effects of SARS- CoV-2 community spread on public and population health, molecular and genomic epidemiology methods are needed. Without the availability of real-time public health surveillance data, a range ofoften-altering pandemic instructions to stop viral spread were implemented globally. Therefore, during the early phases of the pandemic, it became clear that a well-coordinated genomic monitoring network was required to monitor viral propagation and evolution. In developing and low-income countries like Nepal where there is a scarcity of proper resources, facilities, and expertise it was hard to tackle the rampant inflation and surge in cases and establish proper guidelines to carry out molecular and genomic level-based research to keep track of virus spread. To stop the spread of SARS-COV-2, the establishment of home quarantines and entrance and exit health screenings among international travelers were not sufficient. Local and national efforts to limit viral infections were severely hindered by the lack of accurate and evidence-based real-timeSARS-CoV-2 data. Therefore, this study seeks to address these problems by carrying out an RT- qPCR-based molecular approach involving SGTF to presume a contagious variant of concerns circulating in the community like omicron during the third

wave in Nepal, based on 69-70 del mutation in spike protein to further curb its spread in the country.

1.14 Hypothesis

- Null Hypothesis (H0): There is no significant difference between non-SGTF and 69-70 del mutation.
- Alternative Hypothesis (H1): There is a significant difference between non-SGTF and 69-70 del mutation i.e 69-70 del mutation will result in SGTF.

1.15 Objectives

1.15.1 General Objectives:

- To identify the variants with 69-70 del mutation by using TaqPath COVID-19 assay through the S gene target failure (SGTF) approach.

1.15.2 Specific Objectives:

- To carry out the demographic analysis of the patients visiting the Kirtipur Municipality TUBiotech Corona Laboratory during the third wave of COVID-19.
- To sort the covid positive samples based on Ct values of different kits for RNA extraction.
- To carry out automated RNA extraction from covid positiveoropharyngeal/ nasopharyngeal swab samples.
- To differentiate samples with and without 69-70 del mutation based on SGTF results usingTaqPath COVID-19 CE-IVD PCR kit.

CHAPTER 2

LITERATURE REVIEW

In early December 2019, a novel zoonotic coronavirus (CoV) caused a cluster of pneumonia casesin Wuhan, China. The etiologic agent of the 2019 outbreak of pneumonia in Wuhan, China was identified as belonging to the Coronaviridae family and named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) (Market et al., 2020). The acute respiratory disease induced by SARS-CoV-2 is called coronavirus disease 19 (COVID-19) which was declared a pandemic by the World Health Organization (WHO) on March 11th, 2020 (Zhu et al., 2020). As of October 21, 2022, the virus has spread globally with over 630 million cases and over 6 millionfatalities (Worldometer, 2022).

2.1 Mutations and Emerging Variants

Following the discovery of SARS-CoV-2 in China, genetic evolution caused several SARS-CoV-2 novel variants to emerge over time and in various geographical regions (Chakraborty et al., 2022). Like the majority of viruses and diseases, SARS-CoV-2 can mutate to develop forms that the host immune system is unaware of (Koyama et al., 2020; Sanjuán & Domingo-Calap, 2016). The S proteins of coronaviruses frequently experience sequence alterations in their nature,including deletions, mutations, and recombination (Singh & Yi, 2021). World Health Organization (WHO), the Centers for Disease Control and Prevention (CDC), and European Centre for Disease Prevention and Control (ECDC) have classified the emerging variants of SARS-CoV-2 into variants of concern (VOCs) and variants of interest (VOIs) (Chakraborty et al.,2021). The SARS-CoV-2 VOC or VOI is defined as the variant with increased transmissibility, reduction in neutralization by antibodies obtained through natural infection or vaccination, the ability to evade detection, or a decrease in therapeutics or vaccination effectiveness. According to the WHO's most current epidemiological update, five SARS-CoV-2 VOCs have been discoveredsince the start of the pandemic as of December 11, 2021 (Aleem et al., 2022).

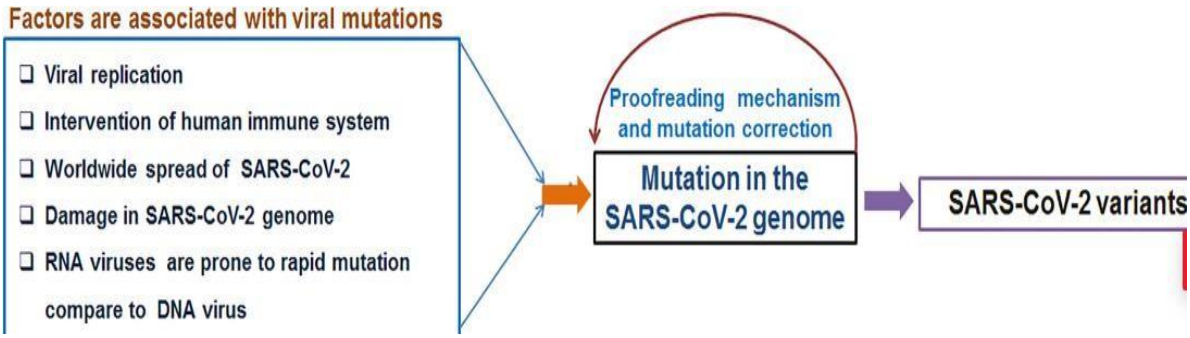

Figure 11: Factors associated with viral mutations (Chakraborty et al., 2021)

Alpha (B.1.1.7 lineage)

In late December 2020, the first highly transmissible variant of SARS-CoV-2, alpha, was reportedin the UK. It has been detected in 94 countries since December 2020. The B.1.1.7 variant was found by genome sequencing as well as in a commonly used commercial test distinguished by the absence of the S gene (S-gene target failure, SGTF) PCR samples. The viral genome of the B.1.1.7 variant carries 17 mutations. Of these, eight mutations (69-70 deletion, 144 deletions, N501Y, A570D, P681H, T716I, S982A, and D1118H) are on spike protein, four mutations on the ORF1ab protein, three mutations on the ORF8 protein and two mutations on the N protein. The spike protein affinity for Angiotensin Converting enzyme (ACE) 2 receptors is increased in N501Y, which facilitates viral attachment and subsequent penetration into host cells (Aleem et al., 2022) (Guo etal., 2021).

Beta (B.1.351 lineage)

A new variant of the SARS-CoV-2 lineage with multiple spike mutations, which resulted in the second wave of COVID-19 infections in South Africa in October 2020 was reported (Tegally, Wilkinson, Giovanetti, et al., 2021). The variant has been detected in 48 countries worldwide by March 2021. The B.1.351 variant carries 21 mutations among which nine mutations (L18F, D80A,D215G, R246I, K417N, E484K, N501Y, D614G, and A701V) are in the spike protein, of which three mutations (K417N, E484K, and N501Y) are located in the RBD and increase the binding affinity for the ACE receptors(Tegally, Wilkinson, Lessells, et al., 2021). According to reports, this variation has a lower ability to be neutralized by post-vaccination sera, convalescent sera, and monoclonal antibody treatment. It also has a higher risk of transmission (Aleem et al., 2022).

Gamma (P.1 lineage)

The third variant of concern, the P.1 variant **was** first reported by the National Institute of Infectious Diseases in Japan on 6 January 2021 in four travelers from Brazil (Guo et al., 2021). This variant harbor ten mutations in the spike protein (L18F, T20N, P26S, D138Y, R190S, H655Y, T1027I V1176, K417T, E484K, and N501Y). Three mutations (L18F, K417N, E484K) are located in the RBD, similar to the B.1.351 variant. According to genetic and mortality data, it is predicted that it may be 1.7–2.4 times more pathogenic and transmissible than prior forms of SARS-COV-2(Aleem et al., 2022).

Delta (B.1.617.2 lineage)

The fourth variant of concern, the Delta variant, was discovered for the first time in India in December 2020 and was responsible for the deadly second wave of COVID-19 infections in Indiain April 2021. The B.1.617.2 variant harbors ten mutations (T19R, (G142D*), 156del, 157del, R158G, L452R, T478K, D614G, P681R, D950N) in the spike protein (Aleem et al., 2022).

Omicron (B.1.1.529 lineage)

The latest SARS-CoV-2 VOC is omicron (B.1.1.529). This variant was first reported to WHO from South Africa on 24 November 2021 (Zawilska et al., 2021). The mutations include T91 in the envelope, P13L, E31del, R32del, S33del, R203K, G204R in the nucleocapsid protein, D3G, Q19E, A63T in the matrix, N211del/L212I, Y145del, Y144del, Y143del, G142D, T95I, V70del, H69del, A67V in the N-terminal domain of the spike, Y505H, N501Y, Q498R, G496S, Q493R, E484A, T478K, S477N, G446S, N440K, K417N, S375F, S373P, S371L, G339D in the receptor binding domain of the spike, D796Y in the fusion peptide of the spike, L981F, N969K, Q954H inthe heptad repeat 1 of the spikes as well as multiple other mutations in the non-structural proteins and spike protein (Aleem et al., 2022).

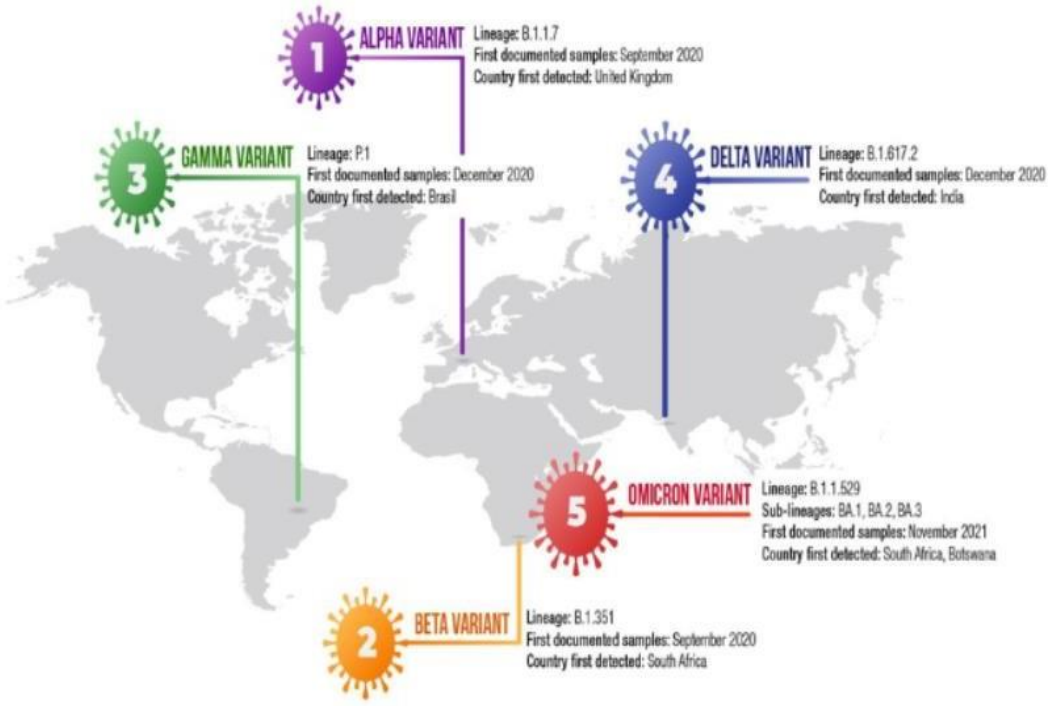

Figure 12: Timeline of SARS-CoV-2 variant of concern (Maki et al., 2022)

2.2 Scenario in Nepal

The Federal Democratic Republic of Nepal is a landlocked country in South Asia that share its border in the North with China, where the COVID-19 disease was first identified, and in the southeast region with India where the cases were growing rapidly (Basnet, Bishwakarma, et al., 2021). The first incidence of COVID-19 in Nepal was discovered in a 32-year-old Nepalese student who had just returned from Wuhan, China, on January 23, 2020. Two months later, on March 23, a second case was confirmed in a person who had entered Nepal from France via Qatar(Pun et al., 2020) (K. Sharma et al., 2021).

There were only two confirmed cases from 610 Reverse Transcription Polymerase Chain Reaction(RT-PCR) tests and no fatalities when the nationwide lockdown was introduced in the first stage.The Government of Nepal issued a nationwide lockdown from 24 March to 21 July 2020, prohibiting domestic and international travel, Indo-Nepal and China-Nepal closure of the border, and non-essential services to check the onset of detrimental effects of the outbreak across the country, which was later eased on 11 June 2020 (Dhakal & Karki, 2020; K. Sharma et al., 2021). On April 4, 2020, the first local transmission of COVID-19 was reported from the Kailali district.On May 16, 2020, the first COVID-19 death was reported. It was of a 29-year-old lactating motherand the second death was reported on 17 May, which

was of a 25-year-old man who was quarantined at Banke district (Basnet, Pant, et al., 2021).

In comparison to the global averages of 2% and 70%, Nepal had a fatality rate and cure rate of 0.71% and 96.97% for COVID-19, respectively. As of January 1, 2021, Nepal had the 44th-highest number of infected cases worldwide and the 15th-highest number in Asia (Basnet, Pant, et al., 2021). Guidelines for the management and handling of quarantine for COVID-19 were finalized on March 26, 2020, by the Ministry of Health and Population (MoHP). In addition, several publichealth measures such as social distancing, handwashing, proper use of masks and hand sanitizers, mass awareness via audio-visual aid, radio jingles, etc. were introduced. Also, a mobile application (Hamro Swasthya) was launched, a separate web portal (covid19.mohp.gov.np) and hotline numbers for COVID-19 were also introduced and various Information Education and Communication (IEC) materials information were disseminated (S. Sharma & Bhatta, 2020).

2.3 S Gene Target Failure (SGTF)

The polymerase chain reaction (PCR)-based method is the gold standard technique used for the detection of SARS-CoV-2 infection (Smith et al., 2022a). During the real-time PCR, the del69/70mutation affects the amplification of the S gene resulting in the S gene target failure (SGTF) (Subramoney et al., 2022). SGTF occurs due to the del69/70 amino acid mutation in the spike gene leading to the failure of probes to bind to the spike gene amplicon. Detection of other amplicon targets, including the nucleocapsid (N) and ORF1ab genes, is un-affected (Guerra-Assunção et al.,2021). But, as the omicron BA.1, BA.1.1, BA.3 and alpha variants result in SGTF due to the shared del69/70 mutations SGTF alone will not uniquely identify the variants although SGTF result is an attainable, fast, and precise indicator for monitoring new variants (Subramoney et al., 2022). Though sequencing on a representative proportion of samples is required to assess the validity or accuracy of the SGTF indicator, the SGTF indication is useful as an initial and quick screening test. (Blomquist et al., 2022).

In December 2020, SGTF was used for the first time as an indicator for variant surveillance for the identification of the Alpha variant. Again, in April 2021, a detectable S gene target with all three targets including the N gene and ORF1ab gene having Ct values <30 was found to be an indicator for the then-emerging Delta variant. Then, in December 2021

SGTF was used for detecting Both Alpha and Omicron genomes which have a deletion corresponding to S protein positions 69 and 70 (Blomquist et al., 2022). Samples with S gene Ct = 0 and N gene Ct < 30 are used to define SGTF. Nonspecific samples are those with S gene Ct = 0 and N gene Ct > 30.1 or S gene Ct > 30.1 and N gene Ct > 30.1. SGTF is not detected in samples with S gene Ct < 30 andN gene C t< 30 (Smith et al., 2022b).

The article by Wolter et al. mentioned that early Omicron wave infections exhibited a lower mean PCR Ct-value, suggesting larger viral loads in Omicron infected patients compared to early Delta infected patients. Also, there was no difference in severity in SGTF-infected individuals and non- SGTF-infected individuals in the same period. In addition, as mentioned in the article, lower risk of severity in SGTF compared to earlier Delta infected individuals, suggested reduced severity may be due to high levels of population immunity by natural infection or vaccination during the omicron wave. The article by Guerrero-Preston et al. mentioned that approximately 95% of the SGTF cases reported by clinical laboratories between December 20, 2021, and March 28, 2022, were later confirmed as Omicron by The Washington State Department of Health's (WA DOH) coordinated WGS sequencing. Therefore, keeping track of SGTF cases help scientists identify community-specific modes of SARS-CoV-2 transmission and help in implementing effective surveillance and preventive measures aimed at quickly identifying and containing viral outbreaks (Guerrero-Preston et al., 2022).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection and site selection

Covid samples were collected from the patients visiting the Kirtipur Municipality-TU Biotech Corona Laboratory, Kirtipur, Kathmandu, Nepal. All the patients who visit the lab for official COVID-19 tests were included in the study. Naso and oropharyngeal swab samples were collectedin a viral transport medium (VTM) and stored in a sample collection box with ice until the samplewas processed. Later, after the RT-PCR, lab collected positive samples were separated from negative ones. The positive samples were sorted from month January 12, 2022 to July 27, 2022 for SGTF analysis which was carried out in Nepal Public Health Laboratory (NPHL) from July 19, 2022 to July 28, 2022.

3.1.1 Sample size

Since the prevalent population of cases was around 980000 according to Ministry of Health and Population (MOHP) data as of 18th June 2022, which was infinitely large, a 95% confidence interval with a 5% acceptable margin of error was considered. The prevalence rate was calculated by dividing total number of covid-19 cases by total PCR tests performed, which gave a sample size of 216. However, the final sample size for the study was 304.

Prevalence Rate= 980000/5700000

=0.17

$$\text{Sample size(n)} = \frac{\text{Statistic for a level of significance(Z)}^2 \text{ X Prevalence(P) X (1-prevalence)}}{\text{Absolute precision(d)2}}$$

$$= \frac{(1.96)^2 \text{ X } (0.17) \text{ X } (1\text{-}0.17)}{(0.05)2}$$

$$= 216$$

3.2 Ethical Approval

The ethical approval was taken from Institutional Review Committee (IRC). The samples collectedfor the lab purpose were used for the research, only after taking written consent

or verbal consentthrough phone calls. All the participants were made clear about the details of the study and privacywas maintained.

3.3 Outline of research

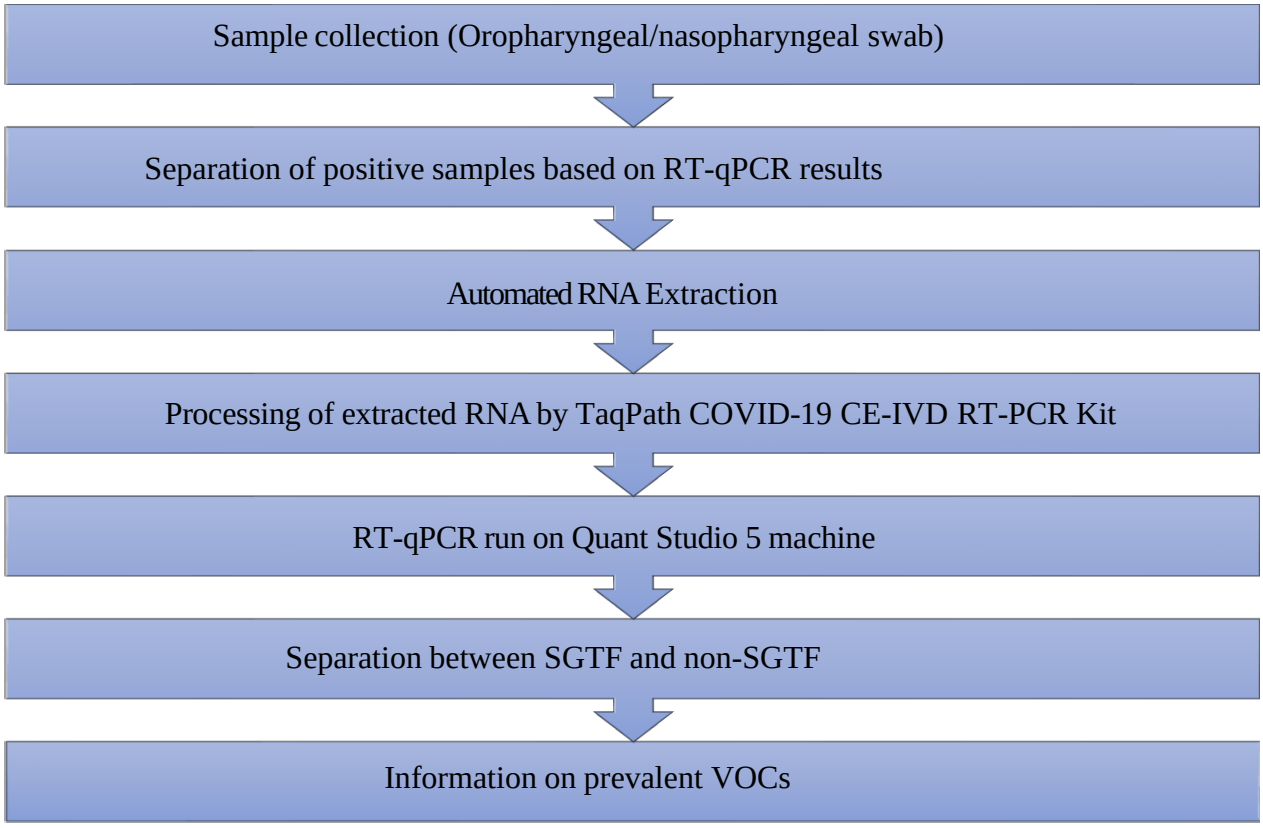

Figure 13: General outline of the research work

3.4 Procedure for the detection of covid positive samples

3.4.1 Upper respiratory tract sample collection

CITOSWAB® Collection and Transport kit were used to collect the sample. Each kit comprises a sterile peel pouch containing a swab, a transport tube containing a medium into which the swab was placed after sampling, and a biohazard bag used to place the transport tube for transportation.Although VTM can maintain organisms for a long time at room temperature it is recommended torefrigerate the specimens at 2-8ºC or kept on dry ice until further processing. In case of a long delay, specimens should be frozen at -70ºC.

The direction used: The pouch was peeled off and a swab sample was collected which was placedinto the medium. The stick of the swab was snapped and the tube cap was screwed

tightly. The information was labeled on the specimen and put into the biohazard bag. The guideline from the Centers for Disease Control and Prevention (CDC) was followed for sample collection (CDC, 2021).

3.4.1.1 Instructions for nasopharyngeal specimen

The patient's head was tilted back at 70 degrees. The swab was inserted through the nostril parallelto the palate (not upwards) until resistance was encountered or the distance was equivalent to that from the ear to the nostril of the patient, indicating contact with the nasopharynx. The swab was gently rubbed and rolled. The swab was left in place for several seconds to absorb secretions. Theswab was removed slowly while rotating it. If a deviated septum or blockage creates difficulty in obtaining the specimen from one nostril, the same swab can be used to obtain the specimen from the other nostril. The swab was placed into the transport tube provided.

3.4.1.2 Instructions for oropharyngeal specimen

The swab was inserted into the posterior pharynx and tonsillar areas. The swab was rubbed over both tonsillar pillars and the posterior oropharynx. Touching the tongue, teeth, and gums should be avoided. Finally, the swab was placed into the transport tube provided.

For collecting specimens from the patients suspected to be infected with SARS-CoV-2, recommended personal protective equipment (PPE) was used, which included a cloth lab coat, disposable gown on top of a cloth lab coat, N95 mask, face shield, shoe covers, head cover and gloves (which were change frequently).

3.4.2 Manual RNA Extraction

For RNA extraction CWBIO Viral DNA/RNA kit was used. Before beginning the extraction process, Washing Buffer 1 and Washing Buffer 2 were prepared by adding isopropanol and 100% ethanol according to the reagent bottle's label and were mixed gently by inverting3-5 times before use. The Biosafety Level 2 (BSL-2) hood was properly sterilized by using 70% ethanol and the ultraviolet lamp was left on for 30 minutes before beginning the extraction process.To begin with the process, 200 µl of lysis buffer, 300 µl of isopropanol, and 300 µl of the samplebalanced at room temperature were added to a 1.5 ml centrifugal tube. The tube was vortexed for15 seconds and kept at room temperature for around 5 minutes. The

tubes were centrifuged at 12000 rpm (~ 13400 g) for 1 minute at room temperature. The filtrate was discarded and the adsorption column was placed back into the collection tube. 500µl of washing buffer 1 (with isopropanol) was added to the adsorption column and was centrifuged at 12000 rpm for 1 minute. The filtrate was discarded and the adsorption column was placed back into the collection tube. 500µl of washing buffer 2 (with 100% ethanol) was added to the adsorption column andcentrifuged at 12000 rpm for 1 minute. The liquid was discarded and the adsorption column was placed back in the collection tube. Dry centrifugation was performed at 13500 rpm for 2 minutes. After the discard of waste liquid was in the collection tube, the adsorption column was placed intoa new collection tube. 40-100µl of RNase-Free Water was added to the middle part of the adsorption column membrane and kept at room temperature for 2 minutes. Final centrifugation was carried out at 12000 rpm for 1 minute and the extracted nucleic acids solution in the collectiontube was stored at -20ºC until the master mix was ready.

3.4.3 Preparation of master mix solution

Based upon the manufacturer's instructions the volume of reagents to be added for master mix preparation differs according to the different kits being used. Uni-medica, BD Biosensor, Foregene, Maccura, and Ardent are some kit names that were used based on their availability at different time periods to carry out the RT-qPCR.

The Uni-medica kit contained 925µl of RT-PCR reaction buffer, 75µl of RT-PCR enzyme mix, and 100 µl of each positive and negative control. Before use, the reagents were thawed completely and short centrifuged. The master mix was prepared by adding 18.5 µl of reaction buffer and 1.5 µl of enzyme mix for a reaction. The volume of the master mix was adjusted based on the number of samples extracted along with one positive control and one negative control. The mixture was short centrifuged and used for the amplification process.

3.4.4 Sample loading

20µl of extracted RNA samples, along with the positive control and the negative control were added to the PCR strip in the respective well containing the same volume of so prepared master mix, resulting final volume of 40µl. The cover of the strip was pressed tightly and centrifuged at 6000 rpm for 10 seconds and transported to the PCR

amplification area.

3.4.5 PCR Amplification

The caped PCR strips were put into the Azure Ceilo™ Real-Time PCR system for amplification. The thermal cycling setting is provided in appendix 1. The FAM channel showed the amplificationfor the ORF1ab gene, VIC for the N gene, ROX for E gene, and Cy5 for the internal control gene. After the completion of the reaction, the PCR strips were disposed of in a sealed bag and treated as medical waste.

3.4.6 Result Analysis

For result analysis, the baseline was set at a region before the exponential amplification, where the fluorescent signals of all the samples are relatively stable. The starting point (cycle number) was set away from the signal fluctuations at the starting phase of fluorescence collection.The endpoint (cycle number) was set 1-3 cycles before the Ct of the first sample to enter the exponential amplification. Similarly, the threshold was set right above the highest point of the negative control amplification curve to nullify the irregular noises interfering with the results.

3.4.7 Quality control and result determination

Before evaluating the specimen results, The Positive Control (PC) and Negative Control (NC) were interpreted using the interpretation table below. If the curve of the positive and negative control is not correct, the sample result will be invalid.

Table 2: Interpretation table for PC and NC

Channels / **Controls**	**Cycle threshold (Ct) value**			
	FAM	VIC	ROX	CY5
NegativeControl	Ct>40 orUNDET	Ct>40 orUNDET	Ct>40 orUNDET	Ct>40 orUNDET
PositiveControl	Ct≤32	Ct≤32	Ct≤32	Ct≤32

3.5 Procedure for S-gene Target Failure (SGTF) Analysis

Based upon the recorded Ct values of the sample for different genes according to the kit used, the positive samples stored at -20ºC were separated from the negative ones and were left to thaw at room temperature. After the samples were thawed completely, it was vortexed for a short time andarranged in the tube holder.

3.5.1 Automated RNA extraction

The biosafety cabinet was decontaminated with 70% ethanol and ultraviolet light was turned on for about 30 minutes before beginning the extraction process. The UV disinfection lamp on the automated Nucleic acid Extraction System from Liferiver™ company was also turned on for the decontamination. 5 µl of Proteinase K was added to the first row numbered from one-twelve in the96 deep-well plates. The extraction machine can extract nucleic acids from thirty-six samples at atime. 200 µl of the sample was added to each sample well. 200 µl of nuclease-free water was addedto the negative control well. 5 µl of MS2 Phage Control from TaqPath™ COVID-19 CE-IVD RT-PCR (Catalog Number: A48067) kit was added to each sample well and negative control well. TheRNA extraction protocol was selected on the automated extraction machine. The plates were loaded in the position as prompted by the instrument and the run button was pressed. After the runwas complete (~22 minutes from the start) the elution plate was removed immediately from the instrument. 50 µl of elution solution was drawn from row ‘E’ for each sample and negative controland added to the newly labeled epitubes. The eluted solution with extracted RNA was kept on iceat -4ºC until the master mix solution was prepared.

3.5.2 Preparation of positive control

To prepare a working stock of 25 copies/µl of TaqPath™ COVID-19 Control ($1X10^4$ copies/µl), 98 µl of TaqPath™ COVID-19 Control Dilution Buffer was pipetted in a microcentrifuge tube towhich 2 µl of TaqPath™ COVID-19 Control was added. The solution was mixed and briefly centrifuged.

87.5 µl of TaqPath™ COVID-19 Control Dilution Buffer was pipetted into a second microcentrifuge tube and 12.5 µl of the dilution created above was added. The solution was well mixed and briefly centrifuged.

3.5.3 Preparation of master-mix solution

The frozen reagents were thawed on ice. The reagents were centrifuged briefly to collect the liquid at the bottom of the tube. As the sample volume was 200 µl during the extraction, 6.25 µl of TaqPath™ 1-step Multiplex Master Mix (No ROX™), 1.25 µl of COVID-19 Real-Time PCR Assay Multiplex, and 7.50 µl of Nuclease free-water per RNA sample or control were prepared in a microcentrifuge tube which was briefly centrifuged and stored at -20ºC until further processing.The final reaction mix volume was 15.0 µl per reaction.

Table 3: Components for master-mix preparation (TaqPath™ COVID-19 CE-IVD RT-PCR Kit - Thermo Fisher, 2020)

Component	**Volume per RNA sample or control**	**Volume for *n* RNA samples plus 2 controls**	**Volume for 94 RNA samples plus 2 controls**
TaqPath™ 1-Step Multiplex Master Mix (No ROX) (4X)	6.25 µl	6.875 × (*n*+2) µl	660 µl
COVID-19 Real e PCR AssayMultiplex	1.25 µl	1.375 × (*n*+2) µl	132 µl
Nuclease-free Water	7.50 µl	8.25 × (*n*+2) µl	792 µl
Total Reaction Mix Volume	**15.0 µl**	**-**	**1584 µl**

Since the RT-PCR was carried out in the different lab, the extracted RNA samples and master-mix solution along with the positive control was transported by sealing them properly with paraffin tape and putting them in a separate zip-lock bag which was finally placed in a box containing packed dry ice with a minimum moment during transport.

3.5.4 Sample loading

In the machine-compatible PCR strips, 15.0 µl of reaction mix solution along with 10.0 µl of extracted RNA was added maintaining the final volume of 25.0 µl. For the positive control

reaction, 15.0 µl of reaction mix solution, 2.0 µl of positive control, and 8.0 µl of nuclease free water were added. The strips were covered tightly with its cover ensuring every well was sealed properly to prevent well-to-well contamination during vortexing and evaporation during PCR. The strips were vortexed for 10-30 seconds to ensure proper mixing and were transferred to the PCR amplification area.

Table 4: Sample and reaction mix volume (TaqPath™ COVID-19 CE-IVD RT-PCR Kit -Thermo Fisher, 2020)

Component	**Volume per reaction**		
	RNA Sample reaction	**Positive Control reaction**	**Negative Control reaction**
Reaction Mix	15.0 µl	15.0 µl	15.0 µl
Purified sample RNA (from RNA extraction)	10.0 µl	-	-
Positive Control (diluted TaqPath™ COVID-19 Control)	-	2.0 µl	-
lease-freeWater	-	8.0 µl	-
Purified Negative Control (from RNAextraction)	-	-	10.0 µl
Total volume	**25.0 µl**	**25.0 µl**	**25.0 µl**

3.5.5 PCR Amplification

The sealed PCR strips were placed in a QuantStudio™ 5 System for amplification. The thermal protocol for time, temperature, and a number of cycles relation is mentioned in

appendix 1. Four different types of reporter dye detected the specific gene; FAM dye detected the ORF1ab, VIC dyedetected the N gene, ABY dye detected the S gene and JUN dye detected the MS2 phage control. Dye channels were defined and targeted in software and applied to the plate.

The program was saved and the export location was confirmed. PCR program was started. When the PCR program was completed, the curves were inspected and the threshold was adjusted for each target, if necessary, to include amplification and discard noise such as background fluorescence. The generated data was automatically saved in the assigned location.

All samples were tested using TaqPath COVID-19 PCR, Thermo Fisher Scientific. SGTF was defined as the non-detection of the S-gene target among samples that tested positive (cycle threshold ≤37) for both the N-gene and ORF1ab gene targets. COVID-19 Assay Ct cutoff values are mentioned in appendix 1.

3.6 Data Analysis

For the statistical data analysis different statistical tools like Chi-square test, Pearson Correlation Coefficient and Mann-Whitney *U* Test was used to check the significant association between the two variables. The value of p at less than 0.05 level of significance ($p<0.05$) was considered significant. Microsoft excel program was used to construct the scatter plot and bar graph for data representation.

CHAPTER 4

RESULT

4.1 Validation of the kit

Laboratory sequenced known variants of COVID-19 sample RNA was separated. The samples included different lineages of omicron variants as it was the circulating variant during the third wave of covid in Nepal. Out of seven samples, three were BA.2 lineage, two were BA.1 lineage and two were BA.1.1 sub-lineage. As BA.1 and its sub-lineage BA.1.1 harbors 69-70 del mutation it resulted in SGTF whereas BA.2 without 69-70 del mutation resulted in non-SGTF validating the procedure by the kit used. Three hundred and four covid positive samples were processed following the same procedure to generate the detailed data. The collected data were analyzed and presented in different figures and tables. The result of the samples used for the validation is mentioned in appendix 2.

4.2 Positive rate

Of the 3,713 samples tested from January 12, 2022 to July 27, 2022, 33.74% (1,253/3,713) of samples were positive. Of the total positive samples, 24.26% (304/1,253) of the samples with lowCt values were selected to access SGTF. The detection rate of SARS-CoV-2 (number of positive tests/number of samples received per month) is shown in figure 14.

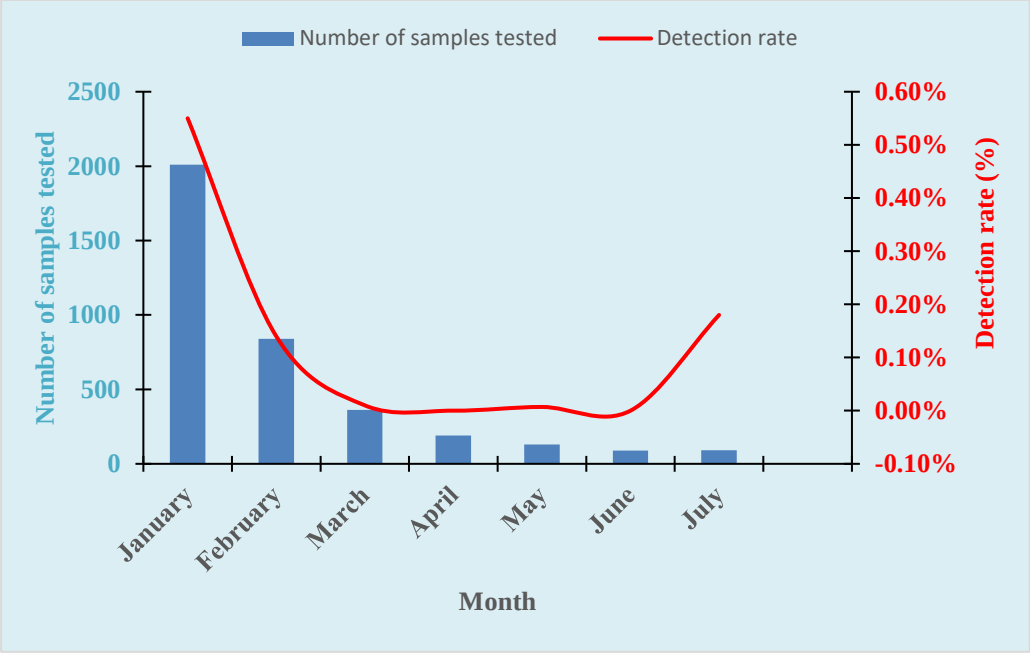

Figure 14: Detection rate of SARS-COV-2

The detection rate of covid was highest in the month of January (0.55%) followed by July (0.18%).There was no positive case of covid-19 in the month of April and June whereas the lowest positive case was identified in May (0.007%).

4.3 Date wise distribution of SGTF and non-SGTF

Highest number of SGTF was observed on 30th January which was 20.40% (10/34) followed by 12.24% (6/43) and 12.24% (6/27) on 27th January and 1st February respectively. Whereas, leastnumber of SGTF was observed on 4th, 7th, 9th February and 13th July which was 2.04%. The trendline shows the decrease in number of SGTF cases with the increasing date.

Table 5: Distribution of SGTF and non-SGTF based on date

Date	Type		Total
	SGTF	**non-SGTF**	
12th January	-	0.39% (1/1)	1
13th January	-	0.39% (1/1)	1
18th January	-	0.39% (1/1)	1
19th January	-	0.39% (1/1)	1
21st January	-	0.39% (1/1)	1
23rd January	-	1.17% (3/3)	3
24th January	-	0.78% (2/2)	2
25th January	-	1.17% (3/3)	3
27th January	12.24% (6/43)	14.50% (37/43)	43
28th January	8.16% (4/39)	13.72% (35/39)	39
29th January	6.12% (3/24)	8.23% (21/24)	24
30th January	20.40% (10/34)	9.41% (24/34)	34
31st January	4.08% (2/21)	7.45% (19/21)	21
1st February	12.24% (6/27)	8.23% (21/27)	27
2nd February	8.16% (4/18)	5.49% (14/18)	18
3rd February	6.12% (3/11)	3.13% (8/11)	11
4th February	2.04% (1/8)	2.74% (7/8)	8
5th February	-	2.74% (7/7)	7
6th February	10.20% (5/10)	1.96% (5/10)	10

7th February	2.04% (1/11)	3.92% (10/11)	11
8th February	-	1.56% (4/4)	4
9th February	2.04% (1/3)	0.78% (2/3)	3
10th February	-	0.39% (1/1)	1
11th February	4.08% (2/3)	0.39% (1/3)	3
14th February	-	0.78% (2/2)	2
15th February	-	0.39% (1/1)	1
17th February	-	0.39% (1/1)	1
19th February	-	0.78% (2/2)	2
23rd February	-	0.39% (1/1)	1
27th February	-	0.39% (1/1)	1
14th March	-	0.39% (1/1)	1
22nd March	-	0.39% (1/1)	1
6th May	-	0.39% (1/1)	1
4th July	-	0.39% (1/1)	1
12th July	-	0.39% (1/1)	1
13th July	2.04% (1/1)		1
14th July	-	0.39% (1/1)	1
20th July	-	0.39% (1/1)	1
24th July	-	1.17% (3/3)	3
25th July	-	1.17% (3/3)	3
26th July	-	1.56% (4/4)	4
27th July	-	0.39% (1/1)	1
Total	**16.11% (49/304)**	**83.88% (255/304)**	**304**

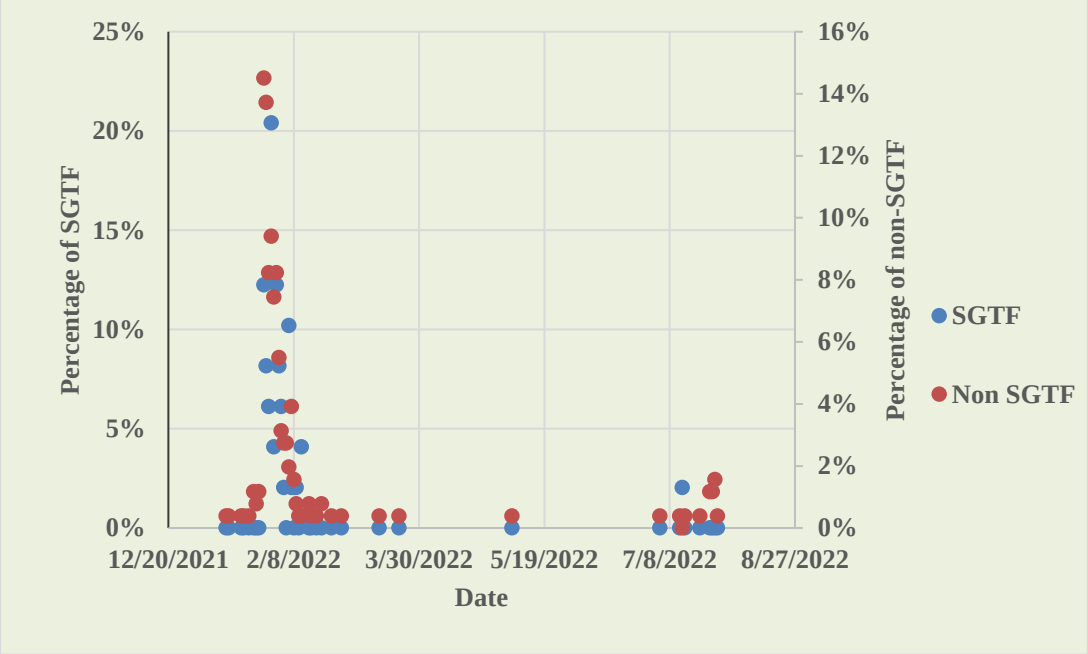

Figure 15: Scatter plot showing date wise distribution of SGTF and non-SGTF

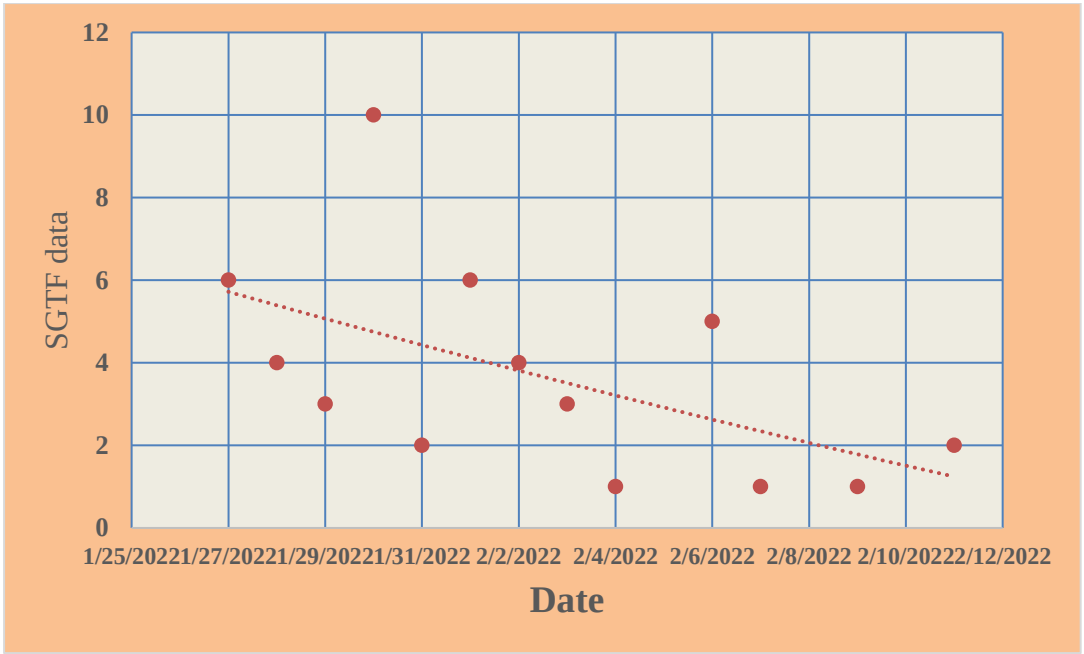

Figure 16: Scatter plot showing date wise distribution of SGTF

4.4 Distribution based on Ct values

Table 6: SGTF categorization based on different range of Ct value of N gene and ORF1ab

Ct value range / Gene	15-20	20-25	25-30	30-35
N gene	2	4	4	39
ORF1ab	4	9	8	28

Highest number of SGTF was found in the sample having the Ct values in the range 30-35 for both N gene and ORF1ab. Whereas only 2 and 4 samples resulted in SGTF which had Ct value in the range 15-20 for N gene and ORF1ab respectively. The amplification curve is shown in appendix 4.

The chi-square statistic was 5.729. The p-value was .125563. The result was not significant atp<.05.

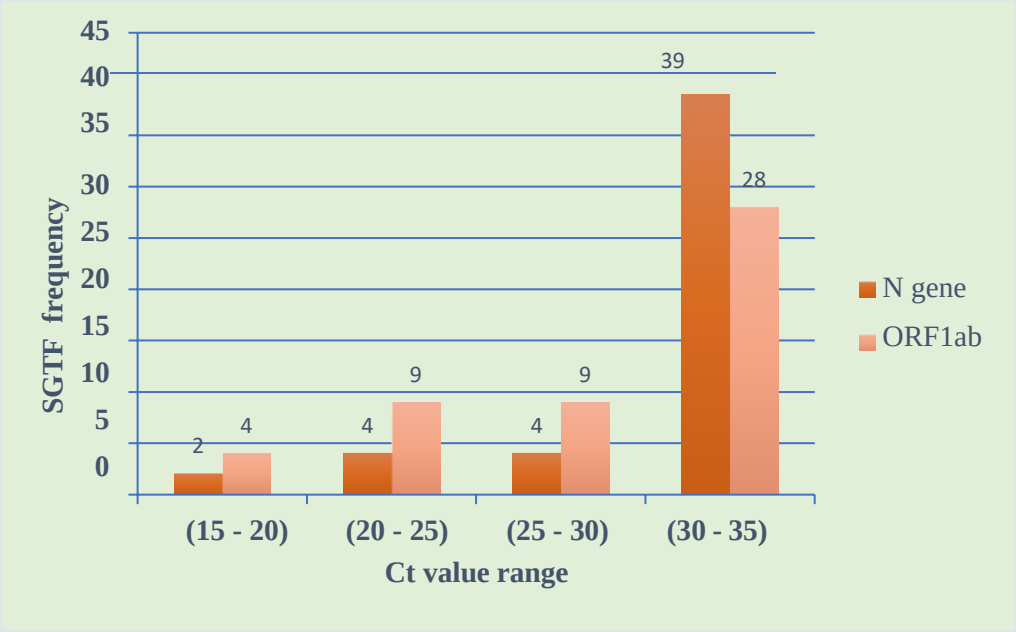

Figure 17: SGTF categorization based on Ct value

4.5 Comparison of Cycle Threshold (Ct) values

Ct mean value of SGTF samples were found to be greater than that of non-SGTF samples. This suggests SGTF is observed in samples with higher Ct values for N and ORF1ab genes.

Type	N gene	ORF1ab
SGTF	Ct mean: 31.28 [16.59-35.49]	Ct mean: 29.03 [16.39-36.19]
Non-SGTF	Ct mean: 23.40 [15.21-35.03]	Ct mean: 22.45 [12.51-35]

4.6 Comparison of Ct values of different genes

N gene and ORF1ab Ct value of 49 SGTF and 255 non-SGTF samples was analysed by using Mann Whitney *U* Test. In both, the median Ct value of SGTF and non-SGTF was significantly different (ORF1ab gene 31.75 vs 21.52; N gene 32.98 vs 22.55; both $P < .0001$, at $p<.05$).

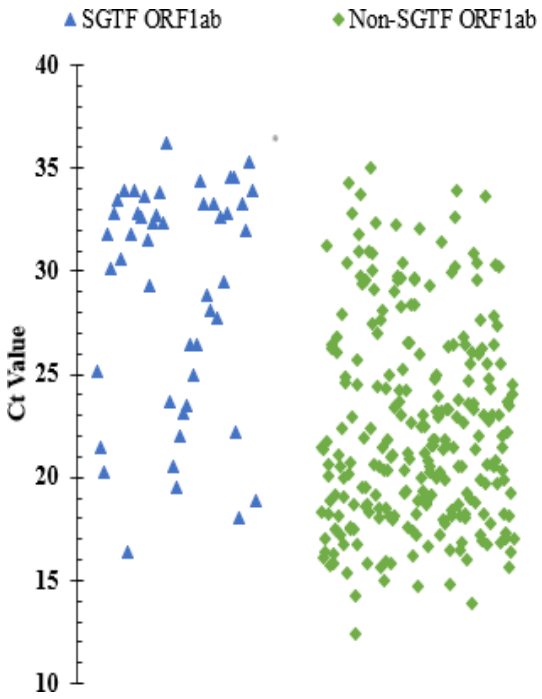

Figure 18: Scatter plot of population of SGTF/non-SGTF Ct value within ORF1ab positive Samples

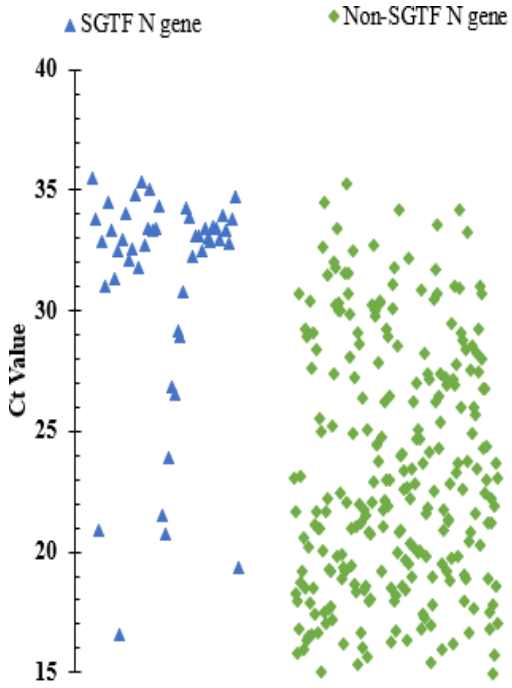

Figure 19: Scatter plot of population of SGTF/non-SGTF Ct value within N gene positive samples

47 Demographic Comparison

4.7.1 Gender wise distribution

Among the samples selected for SGTF analysis, 57.89% (176/304) were male and 42.10% (128/304) were female. Among the 304 samples, 16.11% (49/304) samples resulted in SGTF whereas 83.88% (255/304) samples showed non-SGTF with the amplification of the S gene duringPCR. Among the SGTF samples, 69.38% (34/49) of them were male and 30.61% (15/49) were females. Also, the number of male populations was 55.68% (142/255) and female populations were 44.31% (113/255) among the non-SGTF samples. SGTF and non-SGTF with respective to gender is shown in the figure 15.

The higher number of SGTF cases was observed in male patients compared to female patients (statistically insignificant at $p<0.05$).

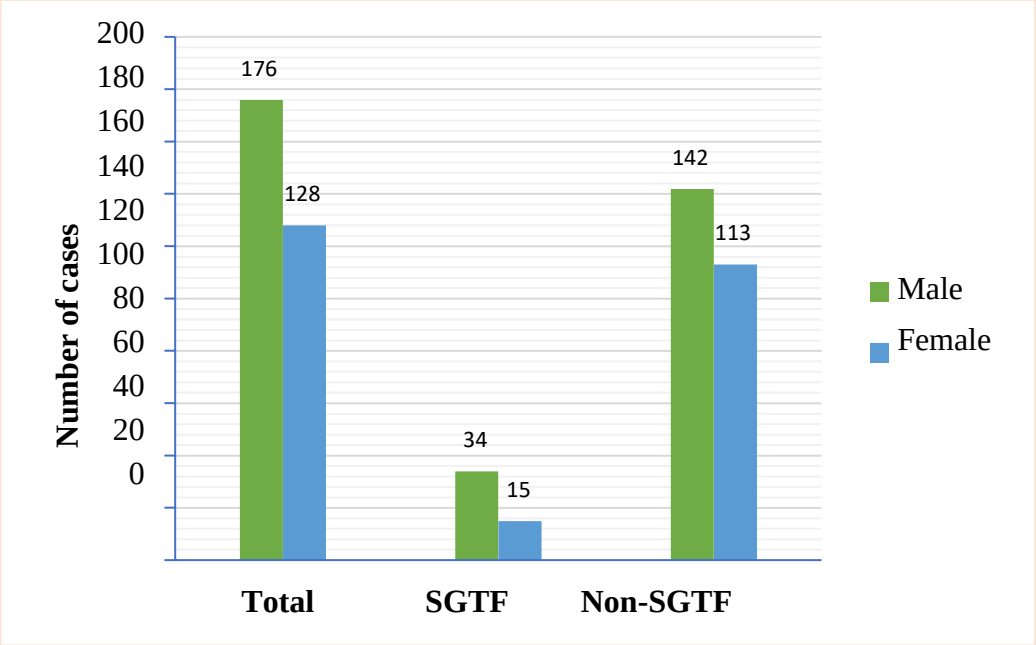

Figure 20: SGTF and non-SGTF with respect to gender

4.7.2 Age wise distribution

The mean age of participants involved in study is 38.20 years ranging from 8 to 73 years old. In the study population highest number of SGTF cases 29/49 (59.18%) belonged to 25-45 years agegroup followed by 45-65 years age group 13 (26.53%) and age group <5 years had lowest number of SGTF cases (2.04%). (r= 0.118, p=0.875; Pearson Correlation Coefficient Test). The result wasnot significant at p<0.05.

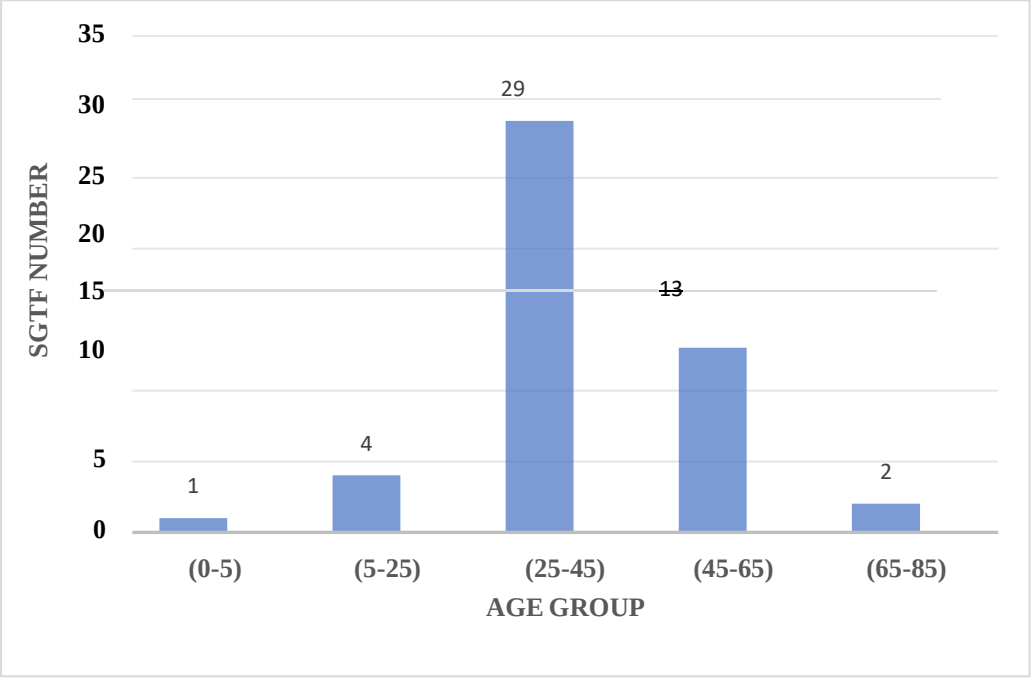

Figure 21: SGTF based on age

4.7.3 Age and gender wise distribution

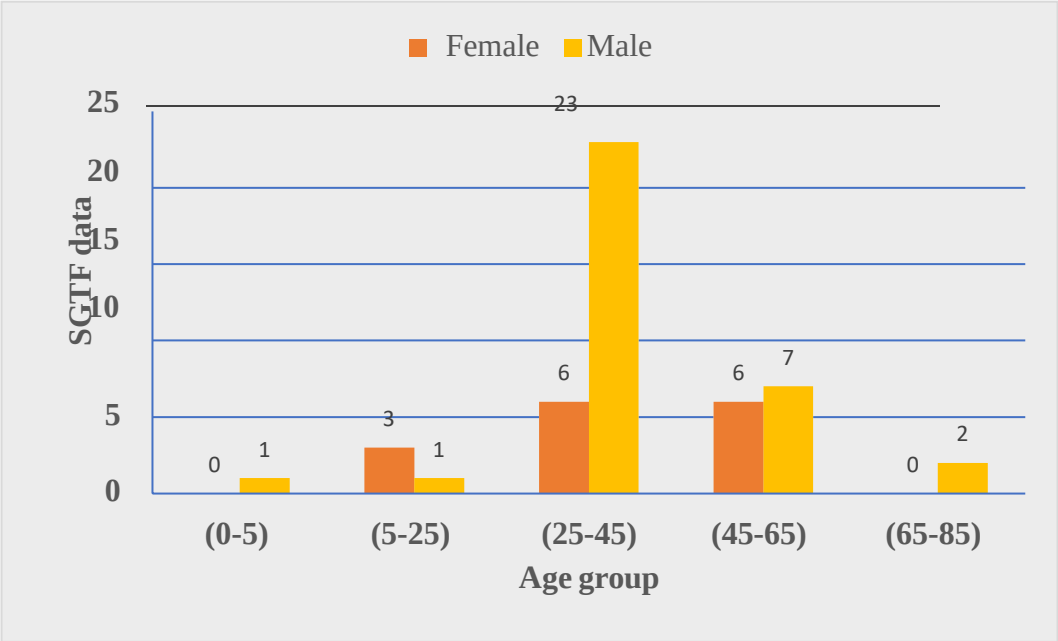

Figure 22: SGTF cases based on age and gender

4.8 Study based on symptoms

The common symptoms experienced by the patients visiting the lab included common cold, fever, cough, headache, sore throat, and body pain. Many of them had combination of these symptoms. Among all the positive samples cough, fever and sore throat were the common symptoms followed by common cold, body pain and headache. Other symptoms like diarrhea and vomiting were less common. 40 samples that resulted in SGTF showed the patients were asymptomatic. Among them, 11 were travelling abroad, 3 had positive contact, 13 were previously positive, 9 of them tested for hospital purposes and 4 of them for retest and had persistent symptoms like weakness, fever, sorethroat and cough.

Table 7: SGTF based on symptoms

Symptoms	SGTF	Non-SGTF	Total
Common cold	1	64	65
Cough	3	81	84
Fever	3	57	60
Headache	1	15	16
Body pain	2	26	28
Sore throat	3	32	35
Diarrhea	-	1	1
Vomiting	-	3	3
Asymptomatic	40	106	146

4.9 Study based on vaccination

Among the SGTF samples the highest number of patients were vaccinated with Verocell followed by Covishield, Johnson and Pfizer vaccines. Two individuals were vaccinated with Astrazeneca and Moderna each. Out of 49 samples, four of them were unvaccinated and one of the patients record on vaccination was missing. Whereas, 2 of them were vaccinated with combination of Astrazeneca and Covishield vaccine.

Table 8: SGTF based on vaccines type

Vaccine type	SGTF	Non-SGTF	Total
Verocell	18	115	133
Covishield	10	49	59
Johnson	6	16	22
Pfizer	6	18	24
Astrazeneca	1	11	12
Moderna	1	2	3
Sinopharm	-	1	1
Unvaccinated	4	13	17
Unknown	1	15	16
Total	**47**	**240**	**287**

4.10 Study based on location

The patients from different geographical locations came for the COVID test in the lab. Highest number of SGTF patients visiting the lab were from Kirtipur Municipality which was 67.34% (33/49) followed by Lalitpur 6.12% (3/49). Whereas, only few numbers of people visited from outside the valley. 2.04% (1/49) from Bardiya, Chitwan, Dailekh, Janakpur, Myagdi, Nawalparasi, Okhaldhunga, Parsa, Ramechhap, Salyan, Siraha, Sunsari and Udayapur each.

CHAPTER 5

DISCUSSION

For real-time reverse transcriptase polymerase chain reaction (RT-PCR) testing methods, S-gene target failure (SGTF) results from the deletion of amino acids 69 and 70 from the spike (S) gene of SARS-CoV-2. According to the study by (Brown et al., 2021b) in areas of England impacted by B.1.1.7, a sharp rise in the proportion of SARS-CoV-2 samples with SGTF was noted. Following validation using whole genome sequencing, SGTF was found to be a valid marker of B.1.1.7 across the country. To quantity the spread of variants with deletion of amino acids 69 and 70 in the S gene, our study tracked the prevalence of SGTF between January 12, 2022 to July 27,2022.

Between January 12, 2022 to July 27, 2022, Kirtipur Municipality TU Biotech Corona Laboratory performed 3,713 SARS-CoV-2 RT-PCR tests using the different COVID-19 assays. Of the 1,253 positive results, 304 samples with low Ct values were separated for SGTF analysis. 49 (16.11%) corresponded to SGTF, as defined by a positive test with non-detectable S gene and cycle threshold (Ct) values of < 37 for N and ORF1ab targets. Whereas, in the study conducted by (Borges et al., 2021; Brown et al., 2021b) out of 36,351 and 20,051 samples that tested positive for COVID-19, 3,367 (9.2%) and 4,692 (23.4%) corresponded to SGTF results respectively.

In our study there was a decline in the number of SGTF cases from 30th January to 9th February with only one case detected after 5 months on 13th July which could be either BA.4 and BA.5 lineage of omicron which was prevalent during the time. But, according to the (Brown et al., 2021a) study done in Canada during the rise of alpha variants in 2021, the number increased from 2.0% to 15.2% within the period of one and half months. Furthermore, it reached 50% at the interval of 15 days. The data showed 1.8-fold weekly increase in SGTF prevalence. Epidemiological evidence suggested the rise in omicron cases in South Africa during December, 2021 along with the rise in PCR test with S gene target failure (Karim & Karim, 2021).

When distribution of SGTF samples based on different range of Ct value for both gene was studied, SGTF sample was found high in number in Ct value ranging from 30-35 and SGTF was found least in Ct value ranging from 15-20. Also, for undetected S gene the mean Ct

value for N gene and ORF1ab was found to be 31.28 and 29.03 respectively. Whereas, for detected S gene the mean Ct value for N gene and ORF1ab was found to be 23.40 and 22.45 respectively. This result was inconsistent with the analysis where higher number of SGTF samples were associated with lower Ct values of ORF1ab and N gene conferring the higher viral load (Kidd et al., 2021). So, higher Ct value in our study may be attributed to the degradation of sample leading to lower viral load due to the storage for a long time. In addition, this study does not investigate the mutations for the N gene and ORF1ab which could have affected their detection resulting in higher Ct values.

In addition to the biological differences various sociological factors including daily activities, jobs, literacy, economy etc plays an important role in gender and age-based susceptibility to diseases (Kushwaha et al., 2020). The highest distribution of SGTF cases of male and female was within 25-45 years age category and the lowest distribution of male and female patients was within 0-5 years age category with male being the dominant gender with high SGTF cases in all the age groupexpect 5-25 age category. The difference between SGTF and age; SGTF and gender categories was found to be statistically insignificant ($p < 0.05$). However, according to the research higher rates of smoking, lower handwashing rates, prior respiratory conditions and biological differencebetween sexes play an important role for higher infection and mortality among males (Betron et al., 2020). Also, the higher infection among females with lower age and males with higher age is relevant with our study as compared to study done by (Kushwaha et al., 2020). In males with the increasing age the adaptive immunity components like CD4/CD8 ratio, B cells, T cell activation weakens which makes vulnerable to infections whereas in female irrespective of age have higher immunoglobulin levels, B cells and antibody response (Klein & Flanagan, 2016). Females also harbor mosaic form of X chromosome resulting in heterogenic ACE2 allele where efficient ACE2 receptor become available in only half of the cells thus limiting the attachment of SARS-CoV-2 virus and increasing protection in females. Whereas, male tends to have increased level of plasma ACE2 concentration which is corelated with immune signatures in lungs of males (Kushwaha et al., 2020).

Symptoms like common cold, cough, sore throat, fever and body aches were more common in patients with SGTF visiting the TU Biotech Corona Laboratory. Although these are the major symptoms, according to research by (Islam, 2022) when people suffer from

flu-like symptoms and illness is mild then they might be infected with Omicron variant attributing to less severity and high transmissibility of the variant. Most of the patients with SGTF were asymptomatic and test was carried out due to positive contact, for hospital purposes, to travel abroad and retest for confirmation. The symptoms like weakness, fever, sore throat and cough were still persistent after 14 days isolation in some patients visiting the lab for retest. Whereas, the mean duration of acute symptoms for Omicron was found to be 6.87 days. It was also directly related to the number of vaccine doses received where mean duration changed to 4.40 days for three doses of vaccines received (Menni et al., 2022). No protective antibody production in the body against SARS-CoV-2 antigen due to no previous infection or vaccination, underlying conditions like high blood pressure, renal failure, diabetes and other comorbidities in immune compromised people, severity of the disease and other risk-factors can lead to long COVID due to persistent symptoms. Also, patients with reinfection can be associated with immune escape mutations present in Omicron (Karim & Karim, 2021).

The increase in rate of infections among the people who were previously infected or have received double vaccines doses has created worries among the nations. New research in Pfizer vaccine claimed that the vaccine protection declined from 96.2% to 83.7% after 6 months. Also, it states double dose provides more than 80% immunity against illness and death and the immunity is increased by 10% by booster dose (Islam, 2022). Most people that resulted in SGTF in our study were vaccinated with Verocell followed by Covishield, Janssen and Pfizer BioNTech. Verocell was found to be less effective in preventing against the infection because most of them had only received double doses without the booster which play an important role in increasing the immunity. Also, the decrease in neutralizing antibodies by the spike mutants results in reinfection and render vaccine to be less effective (El-shabasy et al., 2020).

CHAPTER 6

CONCLUSION

In summary, this research was carried out to determine the efficacy of the S-gene dropout method in differentiating the variant with mutated S-gene and non-mutated S-gene. It is attributed as an accurate and reliable indicator for detecting novel variants. The SGTF cases was higher during thethird wave of covid in Nepal due to the prevalent of Omicron variant with del 69 and 70 mutationsin Spike gene. Detection of new variants of SARS-COV-2 with mutated spike protein through theSGTF approach helps in identifying circulating variants in the community. The emergence of newvariants demonstrates the importance of genomic surveillance in determining viral genetic diversity and the timely identification of new variants for guiding public health intervention measures to check and halt the spread of virus. Identifying new variants is the need in the present context where variants with immune escape and vaccine escape are continuously emerging.

Limitations of the study

- This method only aimed at predicting the circulating variants as omicron or alpha based on 69-70del mutation leading to SGTF without confirmation by sequencing.
- As Omicron has been classified into three sub-lineages, the BA.2 lineage of omicron does not contain the 69-70 del mutation, therefore it could not be identified by the SGTF.
- As the study was completely lab-based and not hospital-based severely infected individuals mighthave been missed to be included in the study.

Recommendation

- S gene target failure combined with genotyping assays targeting the specific mutations would be a reliable indicator in identifying the circulating variant of concerns without the involvement of sequencing.
- Storage of samples for a long period of time will affect the amplification of genes due to the decrease in viral load.
- Study targeting the identification of mutations in the N gene and ORF1ab will help in strengthening the outcome of the study.

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APPENDICES

Appendix 1: Thermal cycle protocol

a. Unimedica

Steps	Temperature	Duration	Cycle
1	50ºC	10 min	1
2	95ºC	3 min	1
3	95ºC	10s	45
	60ºC	1 min	

b. Taqpath

Steps	Temperature	Duration	Cycle
UNG incubation	25ºC	2 minutes	1
Reverse transcription	53ºC	10 minutes	1
Activation	95ºC	2 minutes	1
Denaturation	95ºC	3 seconds	40
Anneal/extension	60ºC	30 seconds	

- **Taqpath COVID-19 Assay Ct cutoff values**

Sample or control	Target	Ct cutoff
Positive Control	MS2	Valid Ct values are >37
	Viral targets	Valid Ct values are ≤37
Negative Control	MS2	Valid Ct values are ≤32
	Viral targets	Valid Ct values are >37
Clinical Samples	MS2	Valid Ct values are ≤32
	Viral targets	Positive Ct values are ≤37

Appendix 2: Validated sample results

Omicron variant lineages	Sample ID	MS2(JUN)	N gene (Hex/Vic)	ORF1ab (FAM)	S gene (ABY)	Results
BA.1.1	Sq 5207	UD	21.51	15.85	UD	SGTF
BA.2	Sq 5224	UD	17.3	17.57	18.84	Non-SGTF
BA.1	Sq 5304	UD	17.45	15.96	UD	SGTF
BA.1	Sq 5449	UD	22.5	20.44	UD	SGTF
BA1.1	Sq 5452	UD	21.2	19.46	UD	SGTF
BA.2	Qc 8321	UD	16.9	18.4	18.86	Non-SGTF
BA.2	Qc 8338	UD	20.31	19.07	20.7	Non-SGTF

Appendix 3: Presentation of RT-PCR results of three hundred and four samples

Date	**LAB ID**	**Thesis ID**	**Lab results**		
			N gene	**ORF1ab**	**S gene**
1/12/2022	BB998	TI1	23.12	21.52	20.37
1/13/2022	CC65	TI2	18.43	18.44	19.01
1/18/2022	CC548	TI3	21.78	21.41	21.87
1/19/2022	CC765	TI4	18.18	16.25	17.47
1/21/2022	DD28	TI5	16.02	16.47	15.76
1/23/2022	DD212	TI6	17.04	17.16	16.37
1/23/2022	DD227	TI7	30.57	31.29	31.05
1/23/2022	DD298	TI8	23.2	21.8	22.78
1/24/2022	DD345	TI9	18.94	18.36	18.77
1/24/2022	DD397	TI10	19.38	20.17	19.46
1/25/2022	DD480	TI11	20.73	20.7	19.51
1/25/2022	DD518	TI12	16.21	15.81	16.21
1/25/2022	DD560	TI13	18.68	18.98	17.89
1/27/2022	DD654	TI14	29.13	26.52	22.39
1/27/2022	DD655	TI15	35.49	25.18	UD

1/27/2022	DD656	TI16	33.81	21.45	UD
1/27/2022	DD659	TI17	28.83	26.31	17.53
1/27/2022	DD663	TI18	16.55	15.98	13.66
1/27/2022	DD664	TI19	20.88	20.3	UD
1/27/2022	DD666	TI20	16.72	16.4	14.65
1/27/2022	DD667	TI21	20.33	19.26	18.56
1/27/2022	DD669	TI22	18.09	17.59	14.72
1/27/2022	DD670	TI23	30.3	26.09	23.06
1/27/2022	DD672	TI24	27.59	26.9	25.61
1/27/2022	DD674	TI25	16.83	17.48	12.77
1/27/2022	DD675	TI26	32.87	31.8	UD
1/27/2022	DD676	TI27	18.65	18.22	17.58
1/27/2022	DD681	TI28	28.99	21.14	17.53
1/27/2022	DD683	TI29	17.67	17.31	17.4
1/27/2022	DD684	TI30	21.22	20.59	19.75
1/27/2022	DD685	TI31	21.76	22.43	20.84
1/27/2022	DD687	TI32	28.32	27.96	24.63
1/27/2022	DD688	TI33	21.06	20.09	19.63
1/27/2022	DD689	TI34	16.91	16.89	16.63
1/27/2022	DD690	TI35	21.07	19.17	17.69
1/27/2022	DD692	TI36	31.01	30.15	UD
1/27/2022	DD695	TI37	25.56	24.62	21.75
1/27/2022	DD696	TI38	24.97	24.96	22
1/27/2022	DD697	TI39	15.3	15.44	14.31
1/27/2022	DD699	TI40	32.43	30.44	22.78
1/27/2022	DD702	TI41	34.46	32.85	UD
1/27/2022	DD703	TI42	20.16	20.27	19.41
1/27/2022	DD704	TI43	34.23	34.29	26.61
1/27/2022	DD705	TI44	17.74	17.62	16.52
1/27/2022	DD706	TI45	21.76	20.84	19.07

1/27/2022	DD707	TI46	17.27	17.66	16.93
1/27/2022	DD710	TI47	31.34	32.8	28.23
1/27/2022	DD711	TI48	22.3	23	21.68
1/27/2022	DD712	TI49	17.89	17.6	17.22
1/27/2022	DD713	TI50	19.47	18.8	18.27
1/27/2022	DD714	TI51	17.97	14.37	12.34
1/27/2022	DD717	TI52	19.37	12.51	18.83
1/27/2022	DD718	TI53	25.24	24.6	24.38
1/27/2022	DD719	TI54	17.39	16.85	15.56
1/27/2022	DD721	TI55	27.36	25.75	25.05
1/27/2022	DD722	TI56	31.84	31.77	32.95
1/28/2022	DD723	TI57	33.36	33.48	UD
1/28/2022	DD724	TI58	31.59	31.01	29.98
1/28/2022	DD725	TI59	30.09	29.78	30.8
1/28/2022	DD730	TI60	33.19	33.76	34.89
1/28/2022	DD731	TI61	31.36	30.58	UD
1/28/2022	DD732	TI62	20	19.61	18.46
1/28/2022	DD733	TI63	30.17	29.42	22.67
1/28/2022	DD734	TI64	29.92	29.54	27.27
1/28/2022	DD736	TI65	22.54	22.02	21.55
1/28/2022	DD738	TI66	29.92	29.64	27.93
1/28/2022	DD739	TI67	20.01	19.61	18.33
1/28/2022	DD741	TI68	19.03	18.7	17.32
1/28/2022	DD744	TI69	19.55	18.76	18.43
1/28/2022	DD745	TI70	16.45	15.97	15.4
1/28/2022	DD746	TI71	19.33	18.42	16.92
1/28/2022	DD747	TI72	31.4	31.03	28.48
1/28/2022	DD748	TI73	22.13	22.45	21.72
1/28/2022	DD749	TI74	35.03	35	34.35
1/28/2022	DD750	TI75	30.58	30.06	32.09

1/28/2022	DD752	TI76	31.36	30.9	30.08
1/28/2022	DD753	TI77	27.99	27.49	26.45
1/28/2022	DD755	TI78	29.74	29.19	28.64
1/28/2022	DD756	TI79	21.08	20.73	20.24
1/28/2022	DD757	TI80	19.61	19.26	18.83
1/28/2022	DD758	TI81	32.26	32.36	30.22
1/28/2022	DD759	TI82	24.93	24.43	23.8
1/28/2022	DD763	TI83	27.15	27.01	25.42
1/28/2022	DD764	TI84	18.87	18.56	17.99
1/28/2022	DD766	TI85	32.46	33.94	UD
1/28/2022	DD770	TI86	16.59	16.39	UD
1/28/2022	DD772	TI87	18.56	18.1	17.13
1/28/2022	DD775	TI88	21.18	20.64	19.97
1/28/2022	DD776	TI89	29.01	27.65	24.93
1/28/2022	DD778	TI90	15.58	15.77	15.5
1/28/2022	DD779	TI91	28.53	28.16	26.12
1/28/2022	DD780	TI92	22.08	21.52	21.33
1/28/2022	DD785	TI93	21.33	20.48	20.23
1/28/2022	DD787	TI94	16.88	15.16	13.41
1/28/2022	DD788	TI95	16.31	16.04	13.88
1/29/2022	DD797	TI96	26.34	24.34	20.81
1/29/2022	DD798	TI97	18.64	18.57	16.2
1/29/2022	DD799	TI98	32.93	31.75	UD
1/29/2022	DD800	TI99	34.02	33.95	UD
1/29/2022	DD804	TI100	21.84	20.43	16.43
1/29/2022	DD806	TI101	21.64	21.87	20.28
1/29/2022	DD808	TI102	18.73	18.19	16.22
1/29/2022	DD809	TI103	25.05	25.01	21.37
1/29/2022	DD810	TI104	15.89	15.9	15.37
1/29/2022	DD811	TI105	20.91	21.07	19.88

1/29/2022	DD812	TI106	18.14	18.04	16.24
1/29/2022	DD813	TI107	20.87	21.14	15.03
1/29/2022	DD814	TI108	18.22	18.19	15.55
1/29/2022	DD815	TI109	32.08	32.78	UD
1/29/2022	DD816	TI110	30.11	29.02	20.57
1/29/2022	DD824	TI111	22.14	23.44	21.93
1/29/2022	DD825	TI112	22.94	23.53	24.02
1/29/2022	DD827	TI113	32.55	32.29	22.56
1/29/2022	DD830	TI114	29.63	29.62	24.77
1/29/2022	DD831	TI115	29.89	29.82	25.25
1/29/2022	DD832	TI116	24.45	24.29	21.84
1/29/2022	DD835	TI117	24.47	23.72	21.17
1/29/2022	DD836	TI118	27.81	28.32	23.71
1/29/2022	DD838	TI119	23.77	23.07	18.65
1/30/2022	DD839	TI120	30.2	29.69	22.91
1/30/2022	DD840	TI121	30.3	30.43	22.97
1/30/2022	DD846	TI122	24.81	25.3	22.85
1/30/2022	DD850	TI123	32.54	32.6	UD
1/30/2022	DD853	TI124	19.39	19.38	20.24
1/30/2022	DD854	TI125	21.84	20.48	22
1/30/2022	DD857	TI126	21.18	19.44	20.73
1/30/2022	DD859	TI127	26.19	24.27	25.33
1/30/2022	DD861	TI128	34.82	33.67	UD
1/30/2022	DD862	TI129	31.79	31.49	UD
1/30/2022	DD863	TI130	22.19	21.3	21.05
1/30/2022	DD864	TI131	35.38	29.26	UD
1/30/2022	DD870	TI132	29.15	26.58	28.2
1/30/2022	DD873	TI133	32.75	32.35	UD
1/30/2022	DD875	TI134	33.38	32.72	UD
1/30/2022	DD877	TI135	35.05	33.87	UD

1/30/2022	DD880	TI136	23.01	17.68	21.31
1/30/2022	DD881	TI137	21.97	20.99	20.12
1/30/2022	DD882	TI138	28.81	26.58	28.4
1/30/2022	DD883	TI139	23.02	22.72	19.61
1/30/2022	DD887	TI140	33.37	32.35	UD
1/30/2022	DD890	TI141	26.44	28.44	24.92
1/30/2022	DD891	TI142	16.5	16.28	17.47
1/30/2022	DD893	TI143	33.42	36.19	UD
1/30/2022	DD895	TI144	18.66	17.47	18.51
1/30/2022	DD897	TI145	29.98	28.42	24.53
1/30/2022	DD899	TI146	30.91	29.61	31.11
1/30/2022	DD900	TI147	31.58	29.73	30.62
1/30/2022	DD902	TI148	18.14	17.36	18.14
1/30/2022	DD903	TI149	18.35	18.29	18.29
1/30/2022	DD904	TI150	16.94	14.86	16.48
1/30/2022	DD910	TI151	20.15	18.93	19.95
1/30/2022	DD911	TI152	34.37	23.71	UD
1/30/2022	DD914	TI153	28.45	26.02	27.75
1/31/2022	DD919	TI154	33.97	32.1	34.34
1/31/2022	DD920	TI155	20.93	20.09	22.06
1/31/2022	DD921	TI156	24.02	22.55	25.74
1/31/2022	DD923	TI157	20.98	19.44	19.16
1/31/2022	DD925	TI158	21.52	20.58	UD
1/31/2022	DD928	TI159	24.13	23.07	23.54
1/31/2022	DD931	TI160	18.76	19.04	17.54
1/31/2022	DD937	TI161	23.39	21.23	24.8
1/31/2022	DD938	TI162	22.69	22.56	20.96
1/31/2022	DD940	TI163	18.58	18.87	18.97
1/31/2022	DD943	TI164	19.82	18.76	20.46
1/31/2022	DD946	TI165	16.55	16.78	16.26

1/31/2022	DD949	TI166	22.72	20.89	21.4
1/31/2022	DD950	TI167	31.97	29.33	30.81
1/31/2022	DD951	TI168	19.63	20.64	20.25
1/31/2022	DD952	TI169	20.76	19.56	UD
1/31/2022	DD956	TI170	20.48	20.34	19.16
1/31/2022	DD958	TI171	22.27	21.67	22
1/31/2022	DD959	TI172	19.17	19.22	18.9
1/31/2022	DD960	TI173	23.47	21.94	21.49
1/31/2022	DD961	TI174	20.34	21.67	21.98
2/1/2022	DD965	TI175	26.23	24.86	27.39
2/1/2022	DD967	TI176	22.92	21.89	23.01
2/1/2022	DD968	TI177	26.95	24.99	26.39
2/1/2022	DD969	TI178	24.73	23.29	23.2
2/1/2022	DD971	TI179	23.88	22.03	UD
2/1/2022	DD972	TI180	20.11	20.09	20.2
2/1/2022	DD973	TI181	25.1	24.03	24.22
2/1/2022	DD975	TI182	24.72	22.97	23.04
2/1/2022	DD976	TI183	20.17	20.33	20.35
2/1/2022	DD977	TI184	17.05	17.33	16.84
2/1/2022	DD978	TI185	30.73	31.48	31.33
2/1/2022	DD979	TI186	23.72	23.29	23.98
2/1/2022	DD980	TI187	17.64	18.03	18.14
2/1/2022	DD982	TI188	28.19	21.44	29.61
2/1/2022	DD983	TI189	17.49	17.88	16.46
2/1/2022	DD985	TI190	19.94	20	17.73
2/1/2022	DD986	TI191	26.87	23.16	UD
2/1/2022	DD987	TI192	26.54	23.51	UD
2/1/2022	DD988	TI193	29.2	26.4	UD
2/1/2022	DD989	TI194	28.95	24.98	UD
2/1/2022	DD991	TI195	21.71	21.89	22.12

2/1/2022	DD992	TI196	21.89	21.6	21.95
2/1/2022	DD997	TI197	27.35	23.39	24.51
2/1/2022	DD999	TI198	24.21	23.37	21.58
2/1/2022	DD1000	TI199	30.76	26.46	UD
2/1/2022	EE06	TI200	27.07	26.36	23.93
2/1/2022	EE07	TI201	15.67	14.9	17.3
2/2/2022	EE10	TI202	17.22	16.54	17.91
2/2/2022	EE11	TI203	19.12	18.27	19.78
2/2/2022	EE19	TI204	34.27	34.4	UD
2/2/2022	EE20	TI205	18.04	18.51	18.78
2/2/2022	EE23	TI206	30.31	29.95	30.02
2/2/2022	EE24	TI207	22.56	21.55	22.56
2/2/2022	EE25	TI208	33.91	33.29	UD
2/2/2022	EE26	TI209	26.17	26.51	26.48
2/2/2022	EE28	TI210	31.57	32.68	33.05
2/2/2022	EE31	TI211	30.54	30.28	29.93
2/2/2022	EE34	TI212	32.23	28.8	UD
2/2/2022	EE37	TI213	33.31	33.88	29.2
2/2/2022	EE42	TI214	26.4	22.97	24.64
2/2/2022	EE45	TI215	33.12	28.14	UD
2/2/2022	EE47	TI216	24.35	23.84	20.26
2/2/2022	EE55	TI217	25.38	20.59	18.58
2/2/2022	EE62	TI218	27.31	18.74	20.99
2/2/2022	EE63	TI219	16.22	16.62	17.67
2/3/2022	EE65	TI220	19.69	18.27	18.47
2/3/2022	EE66	TI221	21.88	20.66	22.3
2/3/2022	EE68	TI222	21.03	16.94	22
2/3/2022	EE80	TI223	27.16	22.69	18.08
2/3/2022	EE81	TI224	27.21	26.89	27.56
2/3/2022	EE87	TI225	26.87	26.48	27.92

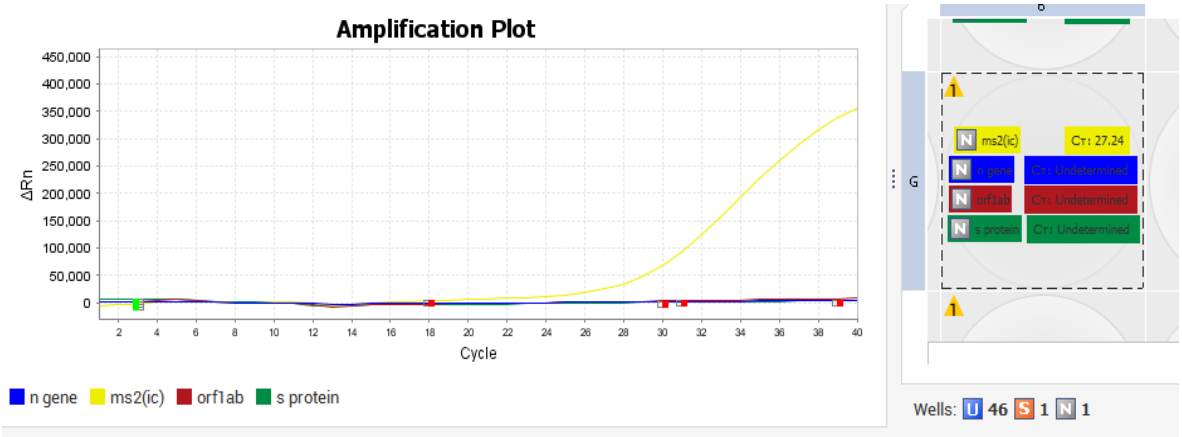
2/3/2022	EE88	TI226	33.12	33.31	UD
2/3/2022	EE91	TI227	32.48	27.7	UD
2/3/2022	EE115	TI228	19.34	18.11	18.35
2/3/2022	EE125	TI229	21.46	18.33	18.73
2/3/2022	EE127	TI230	33.42	32.64	UD
2/4/2022	EE132	TI231	21.38	16.14	18.49
2/4/2022	EE133	TI232	18.98	19.29	17.52
2/4/2022	EE134	TI233	33.04	29.45	UD
2/4/2022	EE143	TI234	22.87	23.66	21.19
2/4/2022	EE147	TI235	29.36	24.75	20.95
2/4/2022	EE148	TI236	19.89	19.02	20.52
2/4/2022	EE150	TI237	27.12	25.59	22.06
2/4/2022	EE152	TI238	16.44	14.02	16.27
2/5/2022	EE165	TI239	26.86	26.17	23.89
2/5/2022	EE166	TI240	30.84	30.91	24.02
2/5/2022	EE167	TI241	23.36	23.39	23.24
2/5/2022	EE171	TI242	27.71	23.65	25.38
2/5/2022	EE175	TI243	19.99	21.45	22.5
2/5/2022	EE179	TI244	23.76	18.89	19.97
2/5/2022	EE182	TI245	33.95	30.4	35.19
2/6/2022	EE187	TI246	32.86	32.77	UD
2/6/2022	EE188	TI247	30.81	29.61	24.59
2/6/2022	EE190	TI248	33.49	34.53	UD
2/6/2022	EE191	TI249	25.98	22.1	24.01
2/6/2022	EE196	TI250	29	26	25.89
2/6/2022	EE199	TI251	33.44	34.61	UD
2/6/2022	EE207	TI252	32.98	22.22	UD
2/6/2022	EE208	TI253	22.64	20	21.05
2/6/2022	EE221	TI254	33.98	18.05	UD
2/6/2022	EE233	TI255	28.72	27.67	26.17

2/7/2022	EE240	TI256	19.19	17.32	18.09
2/7/2022	EE243	TI257	33.35	33.23	UD
2/7/2022	EE247	TI258	18.17	17.07	18.41
2/7/2022	EE252	TI259	28.3	26.32	26.9
2/7/2022	EE253	TI260	19.08	18.33	18.99
2/7/2022	EE254	TI261	23.77	23.09	23.65
2/7/2022	EE256	TI262	33.01	33.67	29.24
2/7/2022	EE265	TI263	20.6	19.75	20.43
2/7/2022	EE266	TI264	16.85	16.83	17.26
2/7/2022	EE269	TI265	27.45	25.61	25.65
2/7/2022	EE285	TI266	20.94	20.68	21.13
2/8/2022	EE296	TI267	24.94	24.83	23.52
2/8/2022	EE303	TI268	28.49	22.78	23.12
2/8/2022	EE307	TI269	17.89	17.88	18.49
2/8/2022	EE319	TI270	26.01	24.37	24.82
2/9/2022	EE324	TI271	25.68	23.12	23.69
2/9/2022	EE335	TI272	21.71	20.67	21.69
2/9/2022	EE336	TI273	32.83	31.99	UD
2/10/2022	EE373	TI274	23.46	20.58	22.47
2/11/2022	EE407	TI275	33.77	35.33	UD
2/11/2022	EE426	TI276	28.16	27.83	28.1
2/11/2022	EE428	TI277	34.76	33.96	UD
2/14/2022	EE499	TI278	27.4	26.46	27.02
2/14/2022	EE502	TI279	29.1	20.94	21.66
2/15/2022	EE521	TI280	30.86	30.32	27.93
2/17/2022	EE567	TI281	20.38	19.77	20.25
2/19/2022	EE591	TI282	27.94	27.42	25.24
2/19/2022	EE595	TI283	30.53	30.29	31.78
2/23/2022	EE702	TI284	24.36	23.06	23.84
2/27/2022	EE792	TI285	26.76	25.58	24.54

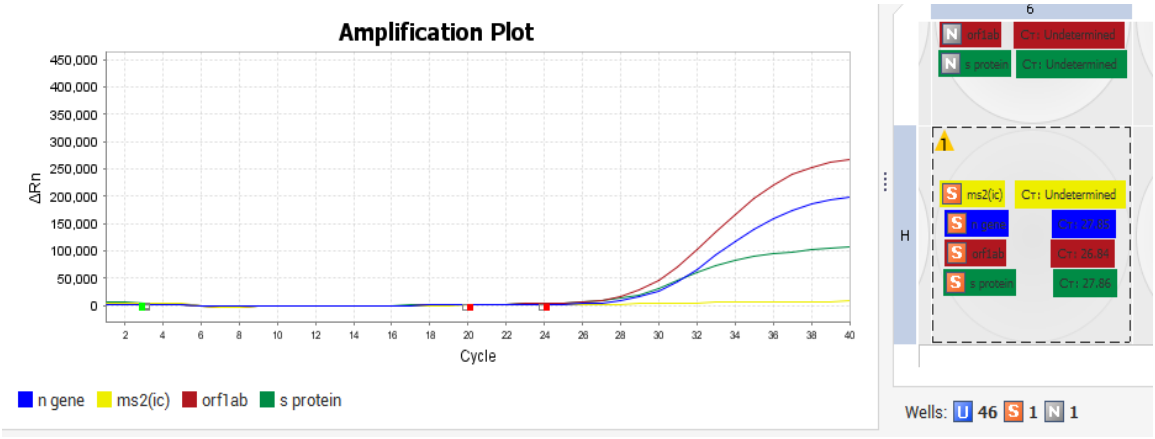
3/14/2022	FF16	TI286	26.72	16.98	15.32
3/22/2022	FF100	TI287	22.55	22.05	20.85
5/6/2022	FF422	TI288	24.43	21.54	18.97
7/4/2022	FF613	TI289	23.02	20.45	18.18
7/12/2022	FF635	TI290	21.33	19.99	17.96
7/13/2022	FF636	TI291	19.36	18.85	UD
7/14/2022	FF641	TI292	19.05	18.3	19.91
7/20/2022	FF650	TI293	17.73	17.16	17.01
7/24/2022	FF666	TI294	17.04	17.45	16.39
7/24/2022	FF675	TI295	22.33	22.28	20.83
7/24/2022	FF676	TI296	21.28	23.7	18.97
7/25/2022	FF687	TI297	15.21	15.8	18.2
7/25/2022	FF688	TI298	17.98	18.23	16.45
7/25/2022	FF690	TI299	21.96	23.58	18.57
7/26/2022	FF693	TI300	15.98	16.53	18.17
7/26/2022	FF694	TI301	18.79	19.32	17.19
7/26/2022	FF695	TI302	23.73	24.54	20.31
7/26/2022	FF696	TI303	23.13	24.11	21.88
7/27/2022	FF699	TI304	17.26	17.15	17.05

Appendix 4: Representation of TaqPath COVID-19 RT-PCR amplification curve

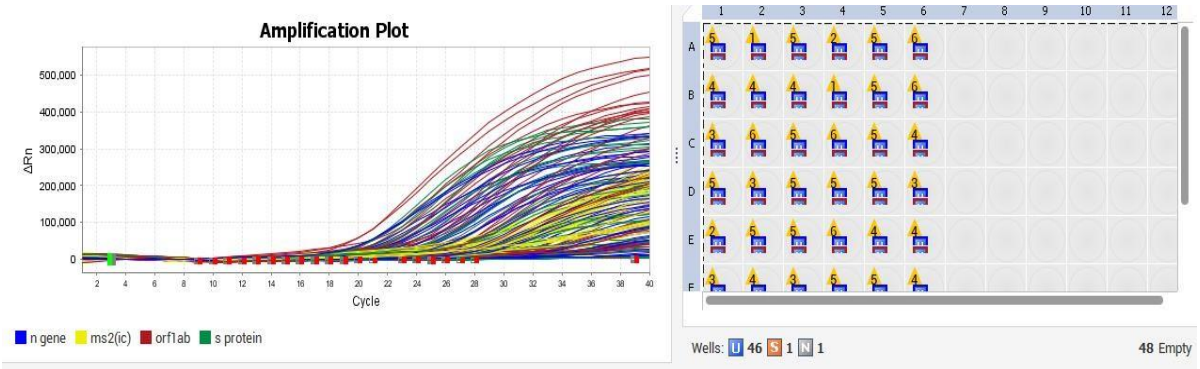
A. Amplification plot of Negative Control and Positive Control



Negative Control

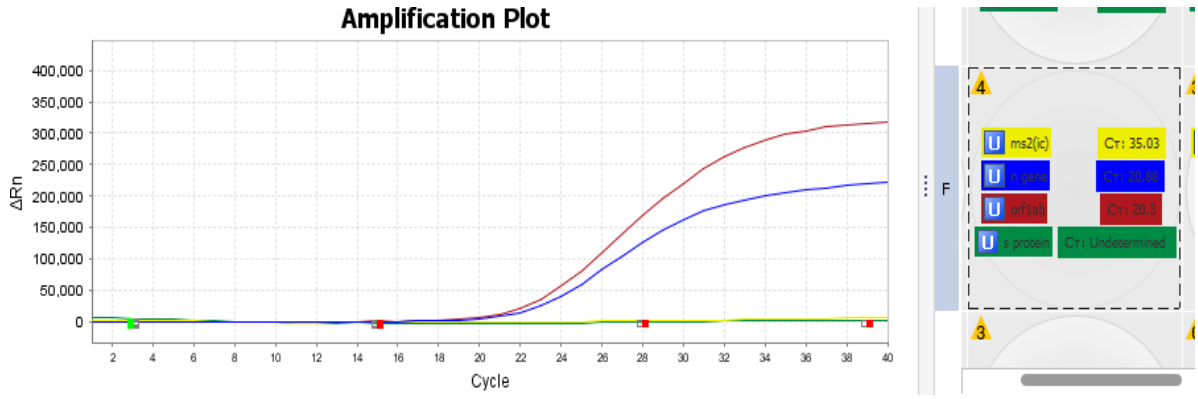


Positive Control

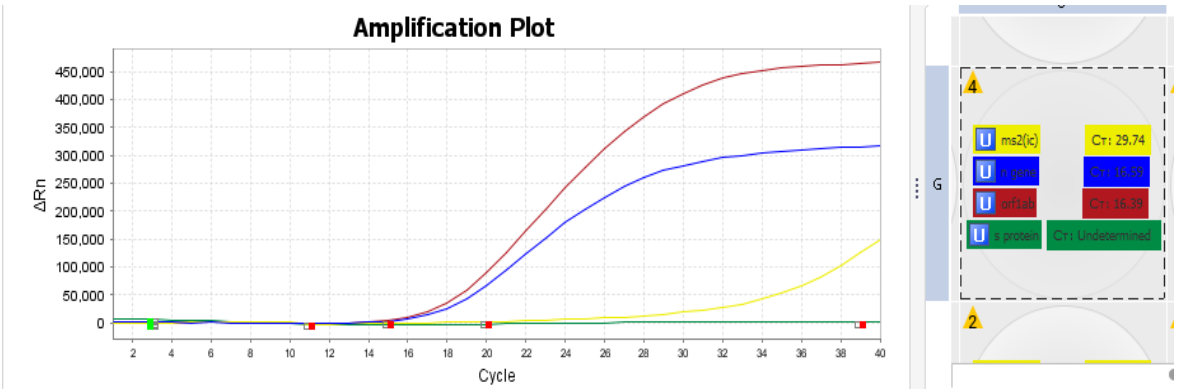


Plot after the completion of PCR

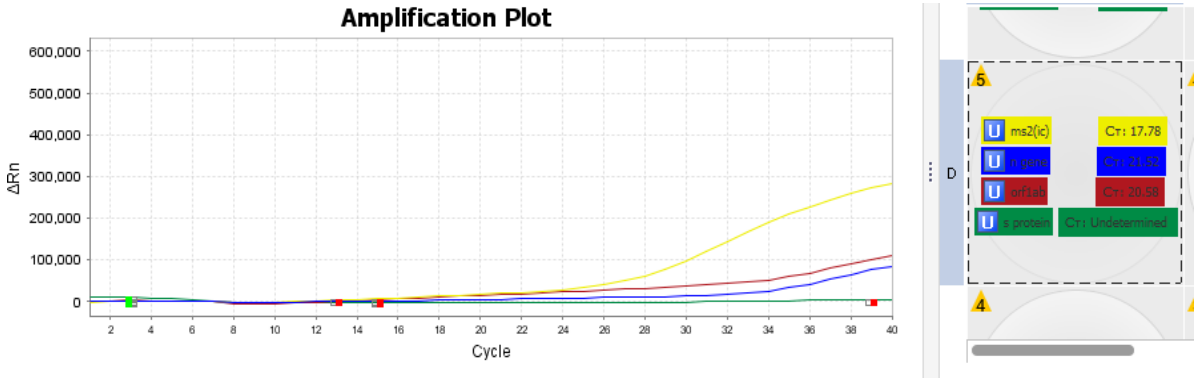
B. Amplification plot of SGTF samples having Ct values less than 30



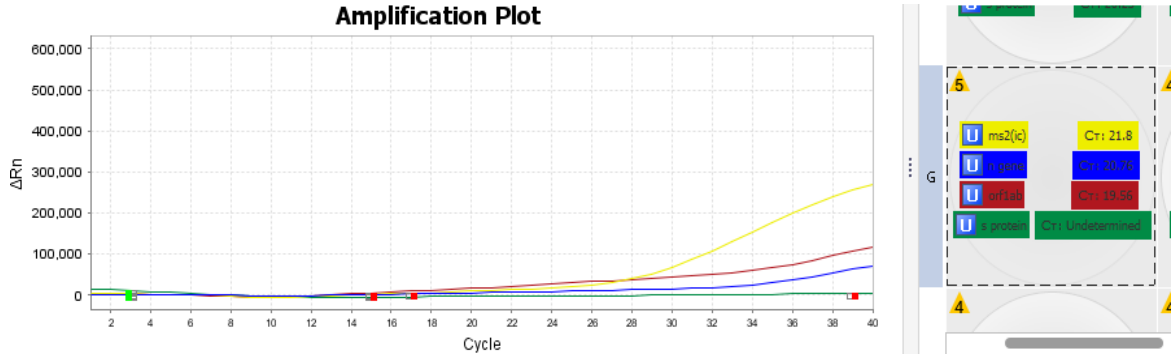
TI 19



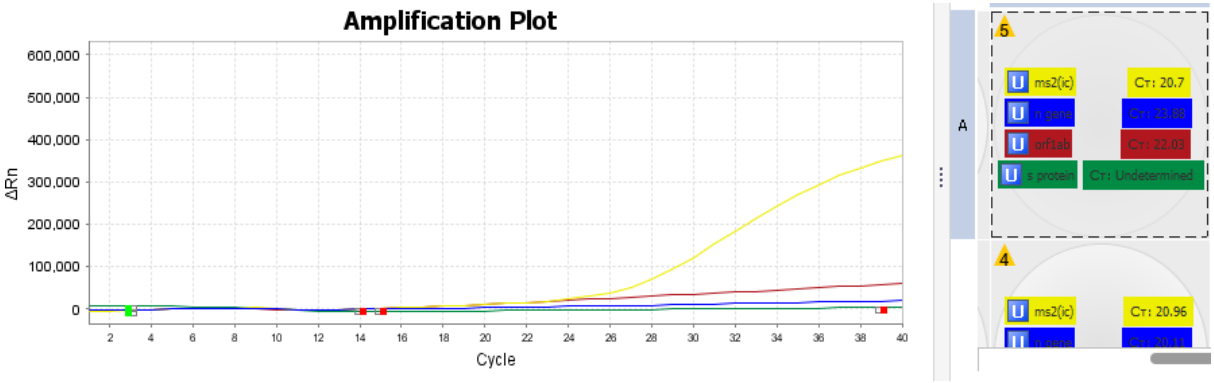
TI 86



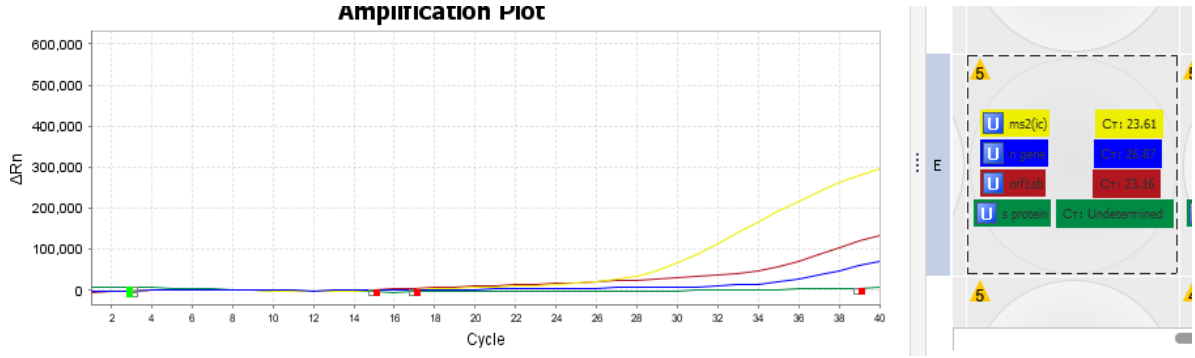
TI 158



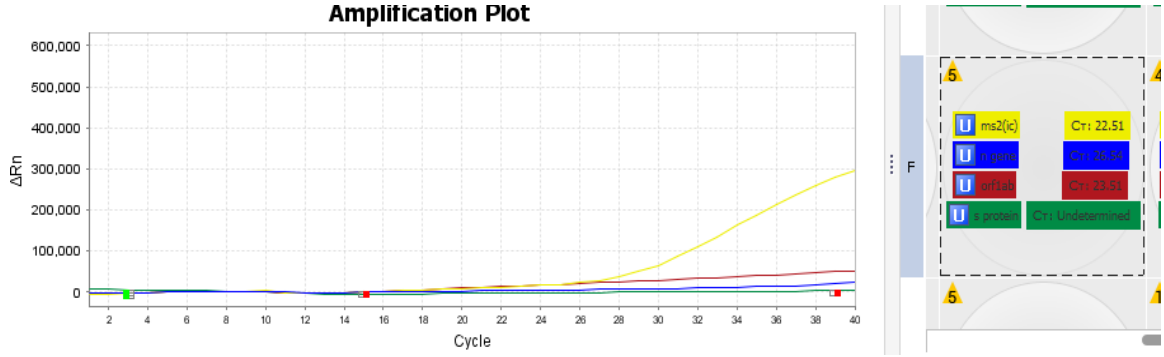
TI 169



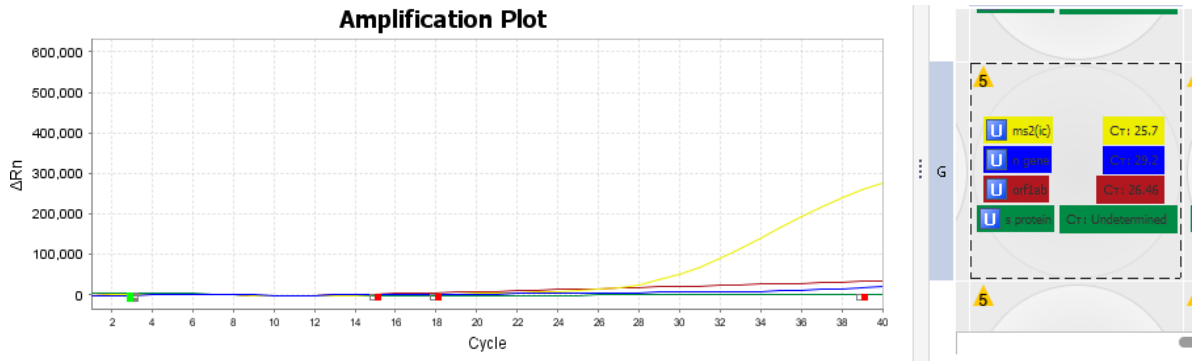
TI 179



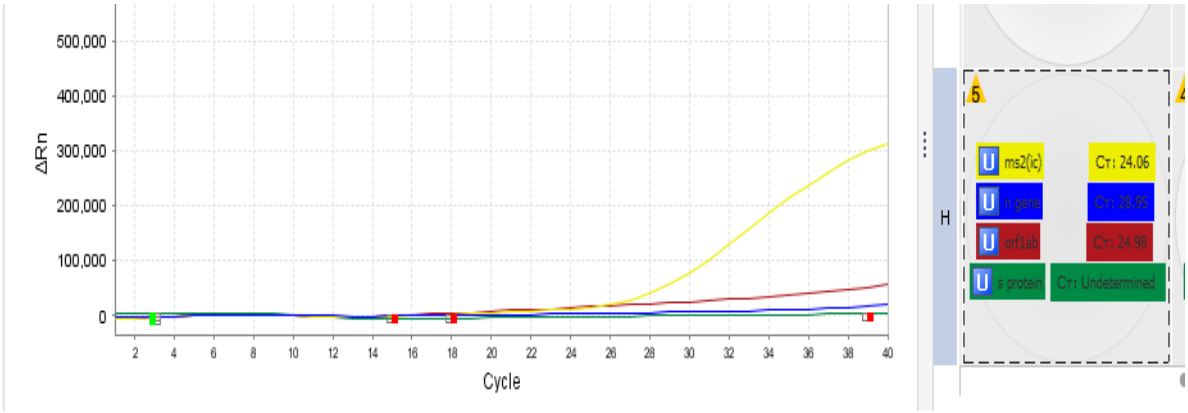
TI 191



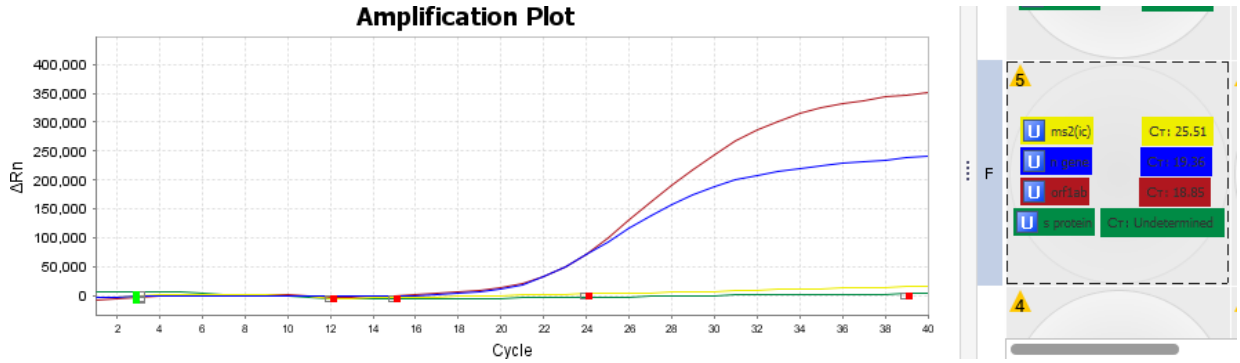
TI 192



TI 193

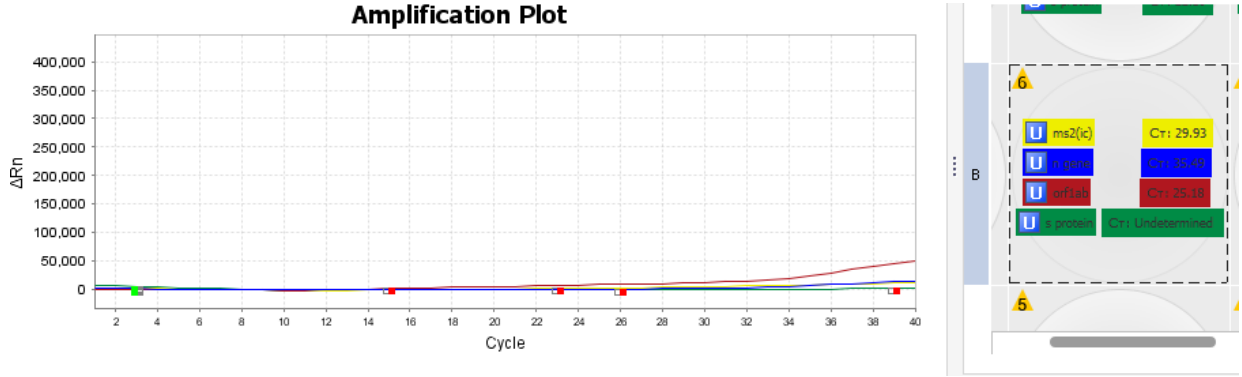


TI 194

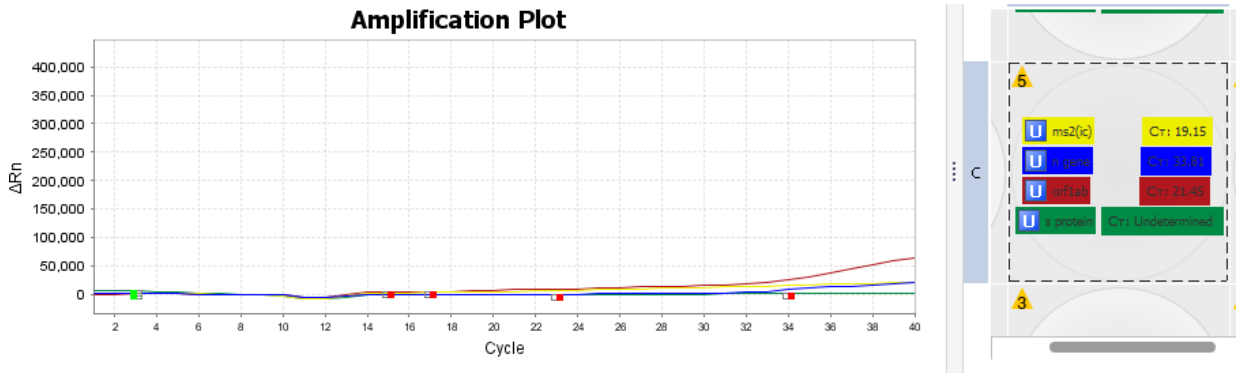


TI 291

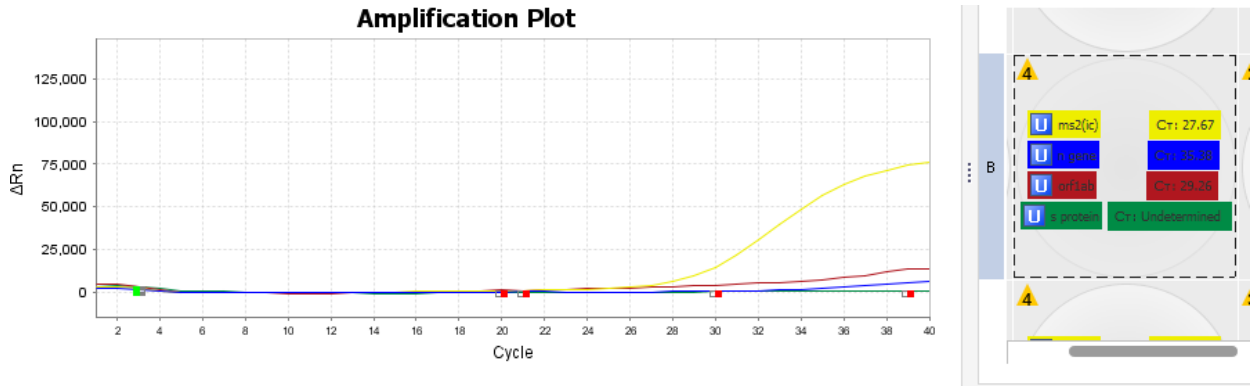
C. Amplification plot of SGTF samples having Ct values of ORF1ab less than 30 and Ngene greater than 30



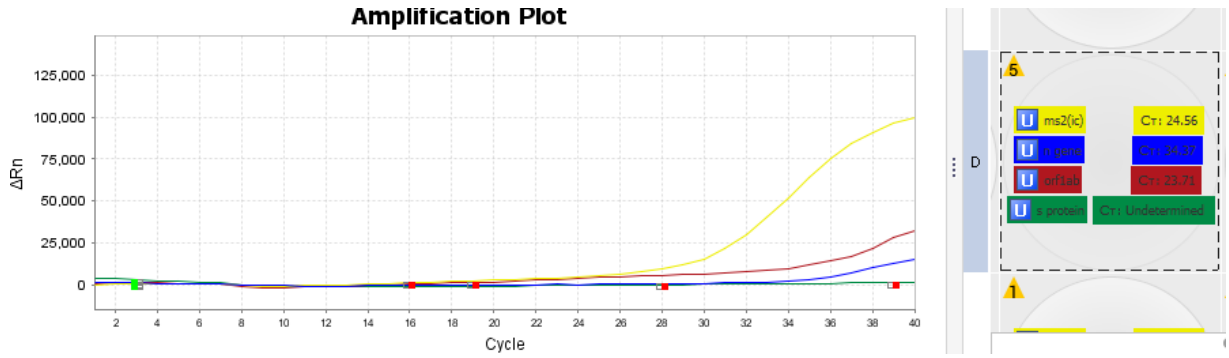
TI 15



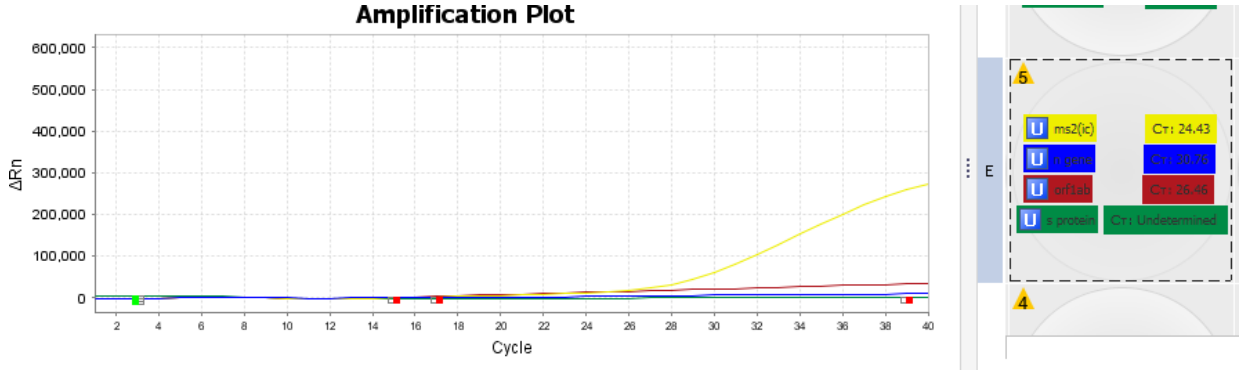
TI 16



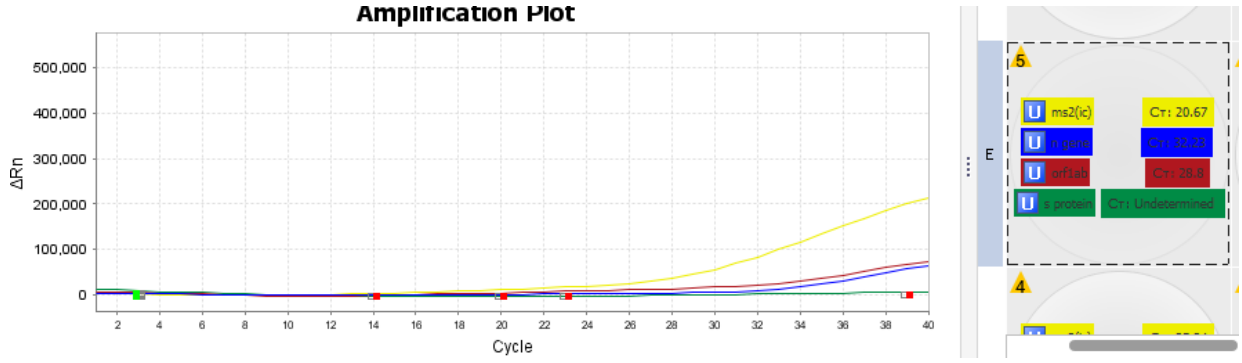
TI 131



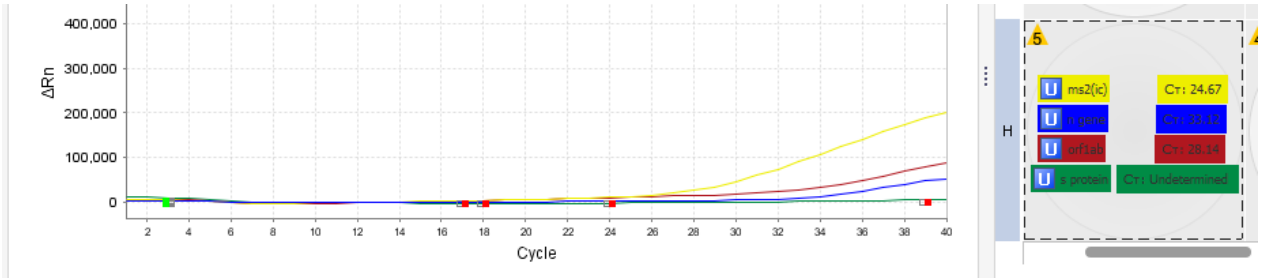
TI 152



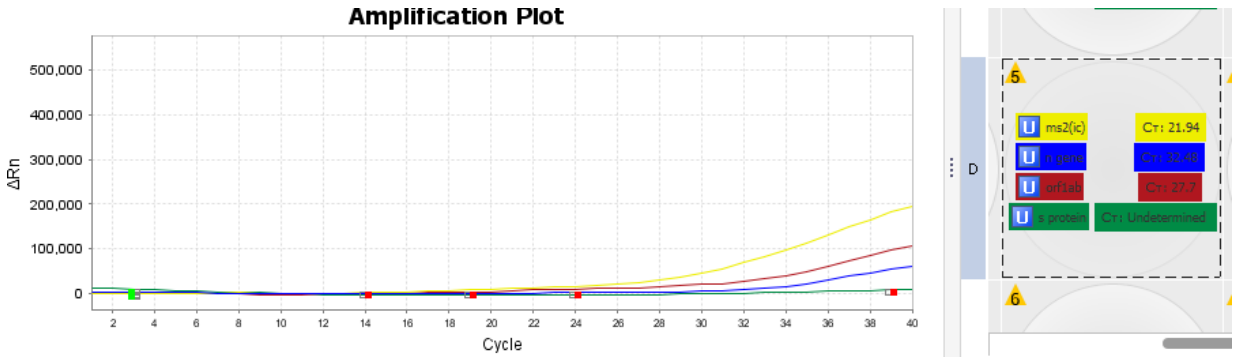
TI 199



TI 212



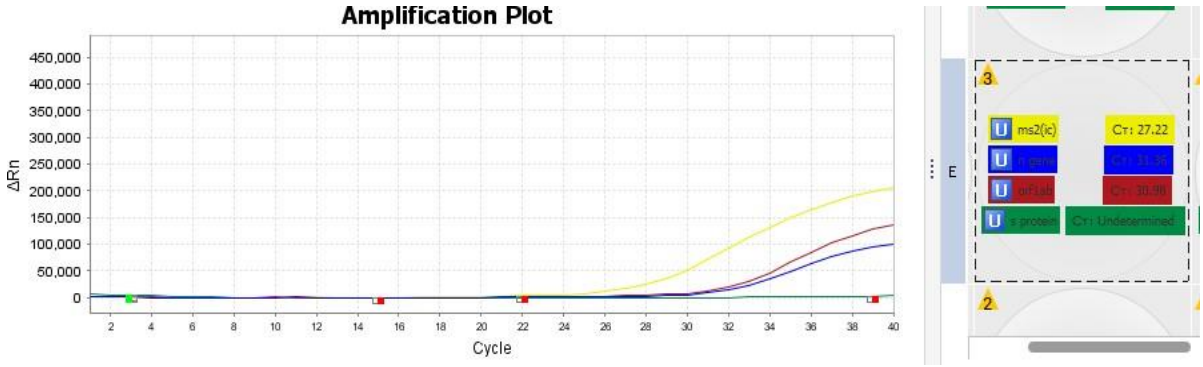
TI 215



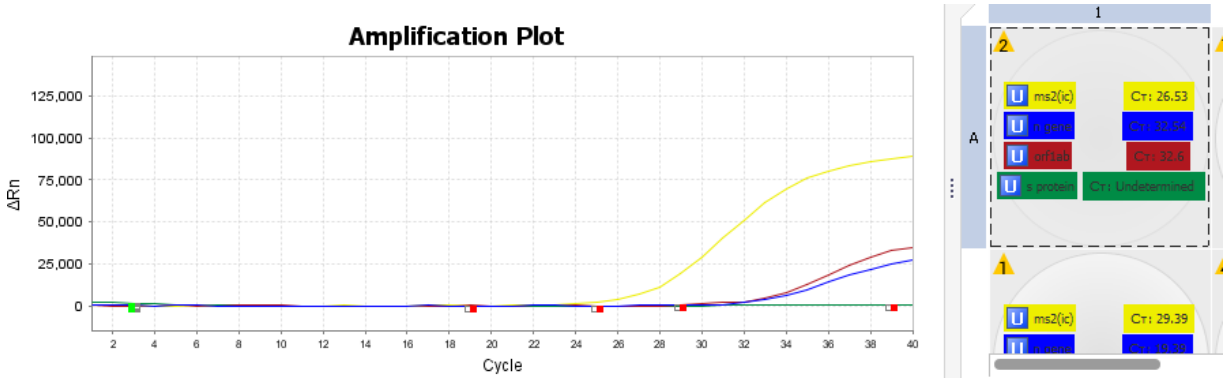
TI 227

D. Amplification plot of SGTF samples having Ct values greater than 30

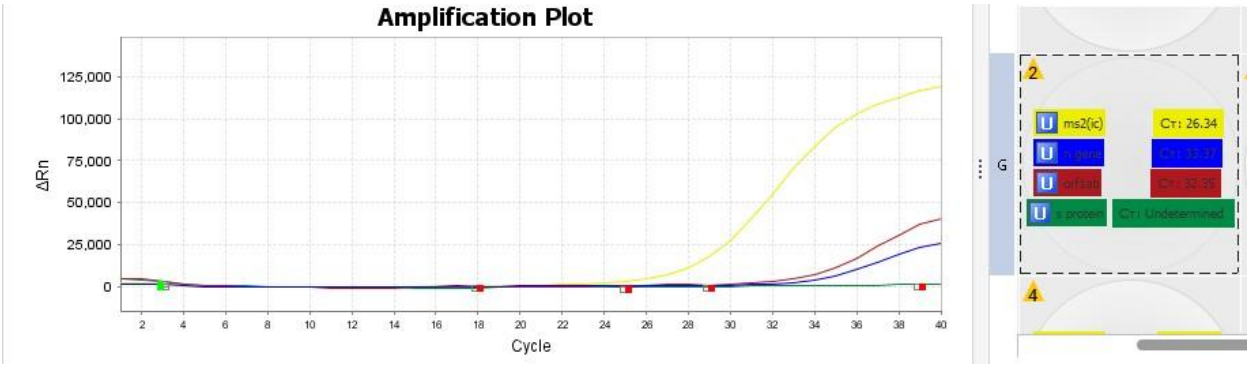
24 samples had Ct values greater than 30 for both N gene and ORF1ab, of which only 5 graphs have been shown below as the pattern of the curve was similar.



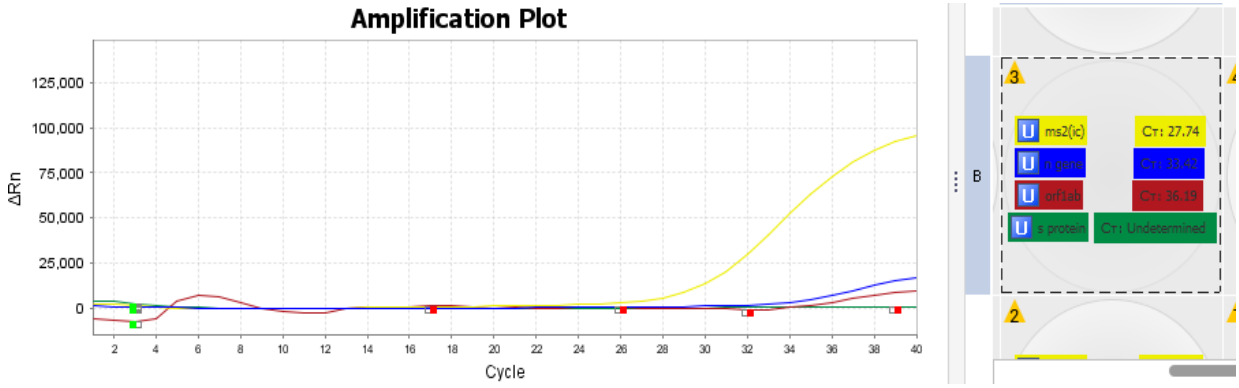
TI 61



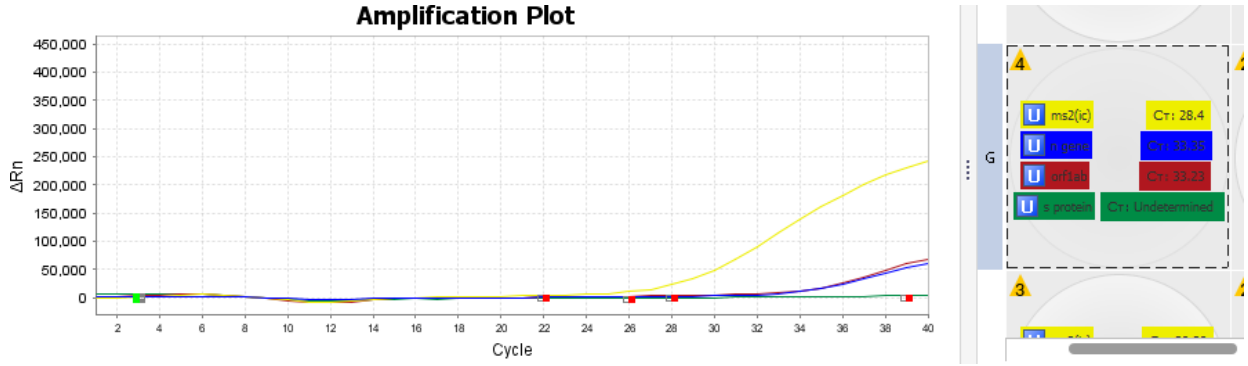
TI 123



TI 140

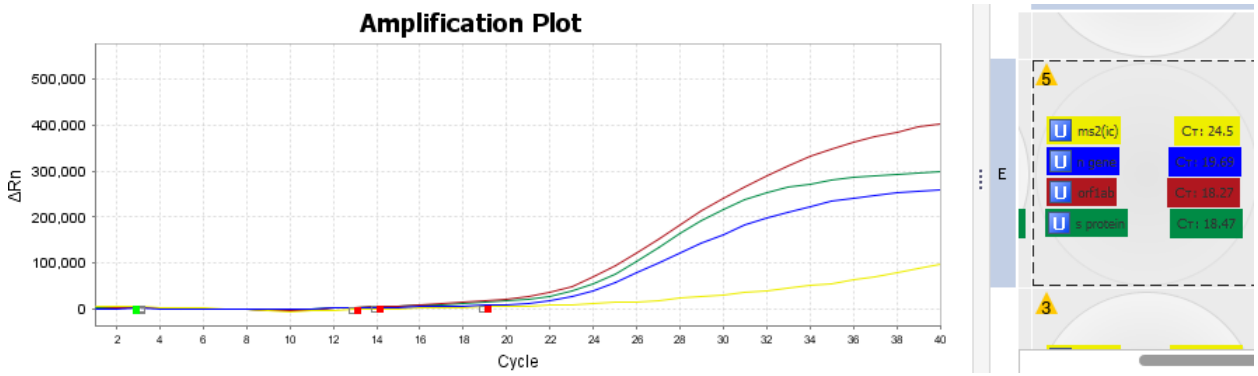


TI 143

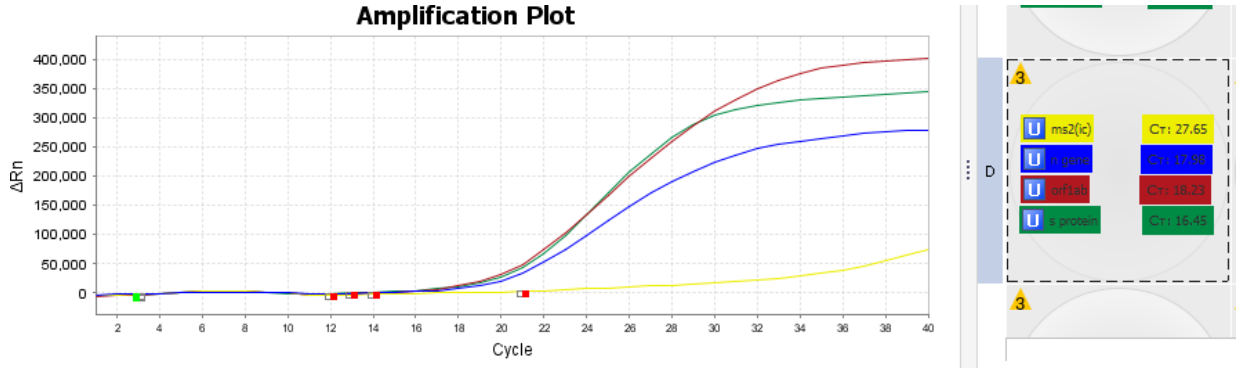


TI 247

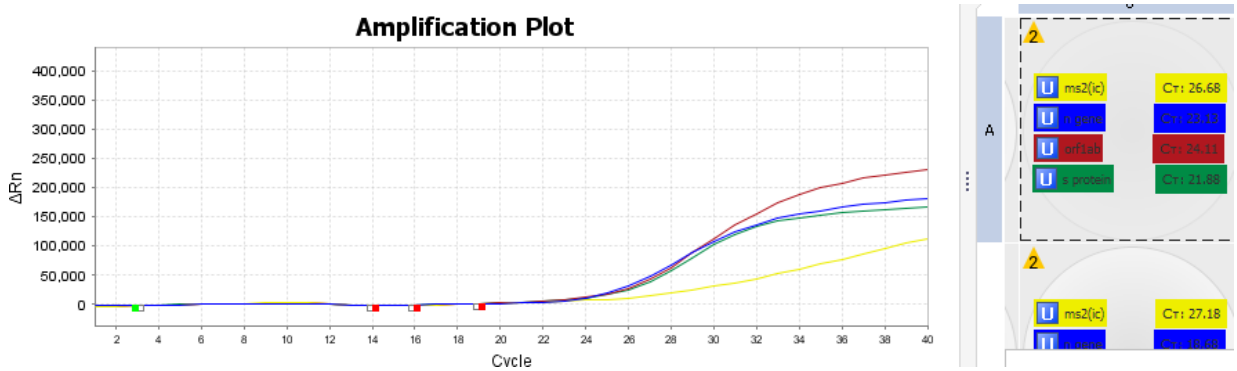
E. Amplification plot of non-SGTF samples



TI 220



TI 298



TI 303

Appendix 5: Ethical Approval and Patient's Consent

Tribhuvan University
Institute of Science and Technology
Kirtipur, Kathmandu, Nepal

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Central Department of Microbiology

Phone: 4331869

Ref. No.: 431078/079 Date: 30 June, 2022

PI: **Prof. Dr. Krishna Das Manandhar**
M.Sc student: **Sushma Acharya**
Central Department of Biotechnology,
Tribhuvan University, Kirtipur

Ref.: IRC Ethical Approval of research proposal entitled **"Detection of SARS-COV-2 Omicron Variant using spike gene target failure and whole-genome sequencing"**

Dear **Prof. Dr. Manandhar,**

It is our pleasure to inform you that the above mentioned proposal submitted on 22 June, 2022 (Regd. No **IRCIOST-22-0044**), following independent expert review and discussion in the IRC/IoST meeting held on **29 June, 2022** has been approved for implementation [start date 30 June, 2022 and end date 29 December, 2022], maintaining ethical principles, set by the Nepal Health Research Council.

The investigators have to strictly follow the protocol stipulated in the proposal. Any change in objective(s), problem statement, research question or hypothesis, methodology, implementation procedure including deviation of the protocol, data management and budget need to be submitted in detail with justification for seeking prior approval to implement the proposed change including extension of the date, in the protocol.

Further, the researchers are also directed to follow the national ethical guidelines published by Nepal Health Research Council during the implementation of research. You are required to submit the final report to the IRC within a month of completion of the research, as planned in the approved proposal.

If you have any questions, please contact the Institutional Review Committee of Institute of Science and Technology, Tribhuvan University.

Thanking you,

Assoc. Prof. Dr. Komal Raj Rijal
Member Secretary
Institutional Review Committee
Institute of Science and Technology
Tribhuvan University

DETECTION OF SARS-COV-2 OMICRON VARIANT USING SPIKE GENE TARGET FAILURE AND WHOLE GENOME SEQUENCING

सम्बन्धि अध्ययनमा सहभागी मञ्जुरीनामा फाराम

विश्व महामारीको रुपमा फैलिरहेको COVID-19 ,कोरोना भाइरसको नयाँ प्रजाति SARS–COV–2 ले हुने भाईरल संक्रमण हो। यो श्वास प्रश्वास, खोक्दा, हाच्छ्युँ गर्दा निस्कने थुक, खकार तथा सिगाँनका कणहरुका माध्यमबाट सर्दछ। माथि उल्लेखित शिर्षक **Detection of sars-cov-2 omicron variant using spike gene target failure and whole genome sequencing** मा नेपालमा हाल कोरोना भाईरस संक्रमणको प्रभाव र प्रकृति बुझ्नका लागि गरिएको एउटा अनुसन्धान हो। तपाईलाई यस अनुसन्धानमा सहभागी गराउनुको मुख्य उद्धेश्य तपाईमा देखिएको स्वास्थ्य समस्या कोरोना संक्रमणले गर्दा हो वा होईन, हो भने यदि तपाई Omicron बाट संक्रमित हुनुहुन्छ वा छैन र तपाईमा यसको संक्रमणकाल कति रहेको छ अध्ययन गर्नु हो।

तपाई यस अनुसन्धानमा स्वयंसेवकको रुपमा आफ्नो नाक तथा मुख बाट तरल पदाथ दिनुपर्नेछ। तपाइबाट लिएको जैविकपदार्थलाई **Kirtipur Municipality-TU Biotech Corona Laboratory** मा विभिन्न साधन प्रयोग गरि अध्ययन गरिन्छ र प्रश्नपत्र (Questionnaire) प्रयोग गरेर पनि तथ्याङ्क निकालिन्छ। तपाईबाट लिइएको जैविकपदार्थ र यसबाट आएको तथ्याङ्कप्रयोग गरि कुनै किसिमको व्यापारिकरण गरिने छैन। तपाईबाट लिइएको जैविकनमुना तथा तथ्याङ्क भण्डार गरेर राखिनेछ र भविष्यमा चाहीएको खण्डमा प्रयोग गर्न सकिनेछ।

फाइदा : यस अनुसन्धानमा सहभागी भएर तपाई वा तपाईको परिवारलाई प्रत्यक्ष रुपमा फाइदा हुन वा नहुन पनि सक्छ। यस अध्ययनमा हुने परिक्षणमा तपाइको शरिरमा Omicron variant को कोरोना भाइरस भए नभएको विषयमा RT-PCR प्रविधि प्रयोग गरि गरिन्छ र यसले रोगको पहिचान गरी संक्रमण कम गर्न मद्दत गर्न सक्छ।

गोपनीयता : यस अनुसन्धान र अध्ययनको नतिजा प्रकाशित गर्न सकिनेछ तर त्यसमा तपाईको नाम तथा परिचय उल्लेख हुने छैन।

यो रोग नेपालमा मात्र नभएर संसारभर नै महामारीको रुपमा फैलिरहेको छ। यसले बेलाबेलामा आफ्नो स्वरुप फेरीरहने र नयां नयां भेरीयन्टको रुपमा फैलिने गरेको छ। त्यसैले तपाईको शरीरमा भाईरसको मात्रा कति छ र उक्त भाईरस निस्कृय हुन कति समय लाग्यो भनी अध्ययन गर्नाले नेपालमा यो रोगको अवस्था थाहा पाउन तपाइको सहभागिताले ठूलो भूमिका खेल्ने छ।

जोखिम : स्वाब लिँदा तपाईले हल्का असहज महसुस गर्न सक्नुहुनेछ।

स्वेच्छिक सहभागिताको बयान : यस अनुसन्धानमा मेरो सहभागिता स्वेच्छिक हो। मैल आफ्नो इच्छाले बिना जरिवाना ,बिना डरत्रास, अनुसन्धानकर्ता समक्ष पूर्व सूचना बिनानै कुनै पनि समय यस अनुसन्धानबाट सहभागिता परित्याग गर्न सक्नेछु। मैले माथि लेखिएका कुराहरु पढेको छु अथवा मलाई माथि लेखिएका कुराहरु पढेर सुनाइएको छ। मेरो प्रश्नहरुको जवाफ दिइएको छ र आफ्नो इच्छाले यस फाराममा सही गरेको छु।

सहभागीको हस्ताक्षर :
(अथवा सहभागीको हकमा अनुमती प्रदान गर्ने व्यक्तिको हस्ताक्षर)

सहभागिको नाम :

ठेगाना :

सम्पर्क नं :

अनुसन्धानकर्ताको नाम :

अनुसन्धानकर्ताको सम्पर्क नं :

अनुसन्धानकर्ताको हस्ताक्षर :

मिति :

Appendix 6: Photos collection