NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

DEPARTMENT OF MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY

TEMPORAL DYNAMICS OF VIRAL LOAD IN RESPIRATORY TRACT SPECIMENS OF COVID-19 PATIENTS IN NORTHERN CYPRUS

M.Sc. THESIS

LUCYANN MUNA AKAH

Nicosia

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Approval

We certify that we have read the thesis submitted by Lucyann Muna Akah titled "Temporal Dynamic of Viral Load in Respiratory Tract Specimens of COVID-19 Patients in Northern Cyprus" and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Medical and Clinical Microbiology.

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Declaration

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Lucyann Muna Akah

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Lucyann Muna Akah

Abstract

Temporal Dynamic of Viral Load in Respiratory Tract Specimens of COVID-19 Patients in Northern Cyprus

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The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) resulted in an unprecedented public health crisis in many countries of the world leading to a global pandemic. Diagnosis of COVID-19 is mainly performed by the reverse-transcriptase real-time polymerase chain reaction (RT-PCR) test due to high sensitivity and specificity. Analysis of RT-PCR data is traditionally performed by estimating the threshold cycle (Ct) at which the exponential phase of the fluorescence signal crosses a baseline threshold. Fluorescence signal and thermal cycles are two semi-quantitative estimates that aid in the categorization of viral nucleic acid in specimens collected from patients following RT-PCR testing. Ct value obtained in an RT-PCR test for the diagnosis of SARS-CoV-2 is inversely proportional to the viral load. Studies have shown low Ct values to be associated with high infectivity. The current study explores the use of RT-PCR to evaluate the Ct values of COVID-19 positive cases diagnosed at DESAM PCR Laboratory at Near East University Hospital and aims to determine fluctuations of the viral load in patient samples due to the emergence of SARS-CoV-2 variants in the Northern Cyprus. For this aim, Ct values of COVID-19 positive samples between January 2021 and October 2021 were analyzed, and Ct values were categorized as <25, 25-30 and >30. The monthly median Ct values were analyzed and a statistical analysis was performed to detect any significant differences in median Ct values (viral load) across months. Results of the study demonstrated higher number of cases in the months of July and August compared to prior months. Compared to January 2021, in which majority of detected SARS-CoV-2 was WT, statistically lower Ct values were observed in March, July, August and September 2021. The positive COVID-19 cases with highest viral load were recorded in the month of July in which SARS-CoV-2 Alpha B.1.1.7 variant was dominant. It is important to note that this coincides with the emergence of SARS-CoV-2 Delta B.1.617.2 variant. This study shows that SARS-CoV-2 Alpha B.1.1.7 and Delta B.1.617.2 variants lead to a higher viral load compared to WT virus. The data collected in this study aids in our understanding of the infectivity timeline and viral transmission, which can further serve as indicator for an upcoming surge.

Keywords: SARS-CoV-2, COVID-19, cycle threshold, viral load, pandemic, surge

TABLE OF CONTENTS

CHAPTER IV

CHAPTER V

CHAPTER VI

List of Tables

List of Figures

LIST OF ABBREVIATIONS

ACE2 – Angiotensin-Converting Enzyme 2

CDC – Center for Disease Prevention and Control

COVID-19 – Coronavirus Disease 19

CT – Cycle Threshold

DNA – Deoxyribonucleic Acid

EA – Expanded Access

EUA – Emergency Use Authorization

FDA – Food and Drug Administration

PCR – Polymerase Chain Reaction

RBD – Receptor Binding Protein

RNA – Ribonucleic Acid

RT-PCR – Real-Time Polymerase Chain Reaction

SARS - Severe Acute Respiratory Syndrome

SARS-CoV-2 – Severe Acute Respiratory Syndrome Coronavirus-2

WHO – World Health Organization

CHAPTER I

1.0 INTRODUCTION

Coronaviruses (CoVs) are enveloped positive-sense RNA viruses. They are members of the subfamily Coronavirinae, and they belong to the order Nidovirales. CoVs are categorized into four important genera that include Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus. They are ubiquitous human pathogens with a diameter of 60–140 nm and single-stranded RNA genome with a 29,903 base pairs. Other characteristics of these viruses include club like spike projections of protein on their surface, with a crown-like appearance which can be observed under an electron microscope (Sharma et al., 2020). This large family of viruses has been found to be associated and well known to cause conditions ranging from the common cold to more acute diseases, such as severe acute respiratory syndrome (SARS). As a result of the emergence and high transmissibility of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), many countries of the world are facing a public health crisis and the entire world has been under the grip of the novel coronavirus, SARS-CoV-2. SARS-CoV-2 was first discovered in Wuhan, China in December 2019 and was declared a global pandemic by the World Health Organization (WHO) in March 2020 due to the exceptional and rapid surge of cases worldwide.

SARS-CoV-2 belongs to the Betacoronavirus genus. It colonizes the human respiratory tract and its symptoms show resemblance to those of common cold. These symptoms include, but are not limited to, sore throat, dizziness, respiratory difficulties, runny nose, cough, head and body aches, malaise, and fever (Mirzaei et al., 2020). At the early stages of infection, infected persons are known to show symptoms of acute respiratory infection. In more severe cases, these symptoms expeditiously develop into acute respiratory failure and other serious complications (Zheng, 2020). The most common mode of transmission is from person to person primarily through droplets of aerosol particles (Song et al., 2020). Certain preventative protocols have been put in place to avert the spread and curb transmission, these include the of face masks

wearing, washing hand for a minimum of twenty seconds as frequent as possible, limiting human contact particularly when certain symptoms can be observed, avoiding public spaces and quarantine (Khailany et al., 2020).

For critical control of the sources of infection, prevention of transmission and disease progression accurate prediction of further infection hotspots, identification and detection of SARS-CoV-2 and its circulating variants across geographical regions is pertinent as well as time sensitive. This is also important for the development of vaccines and diagnostic tests as the high transmissibility and increasing number of COVID-19 cases has made it exceedingly urgent. All this can be achieved by the complete the sequencing and analysis of the viral genomes, which will result in a comprehensive view of all genetic variants at once. Rapid and reliable nucleic acid detection-based techniques been developed for viral detection. Due to its high sensitivity, specificity, and rapid detection ability, polymerase chain reaction (PCR) method amongst the nucleic acid tests has become a gold standard for the detection of viral agents. Real-time PCR (quantitative PCR, qPCR) is a well-established method used in the detection, quantification, and typing of different microbial agents. Real-time PCR (RT-PCR) has been found to be exceedingly useful throughout the pandemic for the detection of SARS-CoV-2 due to its benefits as a specific and simple qualitative assay (Shen M, 2020). Furthermore, RT-PCR has been found to have adequate sensitivity to aid in the diagnosis of an early SARS-CoV-2 infection.

RT-PCR has been extensively exploited worldwide since the beginning of the pandemic which detects the SARS-CoV-2 viral RNA signatures to confirm the presence of SARS-CoV-2 in a patient sample. RT-PCR combines PCR amplification chemistry with detection of amplified products with fluorescent probe for determining the presence of pathogen-specific genetic material. The analysis of RT-PCR data is traditionally performed by estimating the threshold cycle (Ct) at which the exponential phase of the fluorescence signal crosses a baseline threshold. Fluorescence signal and thermal cycles are two semi-quantitative estimates that aid in the categorization of viral genetic material in specimens that have been collected from patient following testing by RT-PCR. Fluorescence signal and thermal cycles can both vary and are classified as low, medium, or high. In a standard RT- PCR assay a maximum of 40

thermal cycles are ran (Kashya et al., 2020). A low Ct represents a high concentration of genetic material, which is an indication of high infection risk (Rao, 2020). A high Ct value represents low concentration of viral genetic material, which is an indication of a lower level of infectivity risk. There is an inverse correlation between Ct values and the viral load. Lower Ct value may be an indication of a severity of infection.

The current study aims to evaluate the Ct values obtained from RT-PCR testing of COVID-19 positive individuals performed at DESAM COVID-19 PCR Testing Laboratory at Near East University Hospital in Northern Cyprus between January and October 2021. By determining the viral load in respiratory tract specimens of COVID-19 patients per month, the possible effects of the emergence of SARS-CoV-2 variants on the viral load will be evaluated. This study will provide towards a better understanding of patient viral load and infectivity timeline and effects of variants which will be significant in monitoring disease progression, as well as predicting and hence preventing a surge during the pandemic.

CHAPTER II
LITERATURE REVIEW

2.0 ORIGIN OF SARS-CoV-2

SARS-CoV-2 is the ninth coronavirus known to infect humans. In the last two decades, several coronaviruses have been documented (Lednicky et al., 2021; Vlasova et al., 2021). A large percentage of previously documented human coronavirus have been known to possess zoonotic origin, this also includes most of the human viruses. SARS-CoV-2 shares numerous characteristics with preceding zoonotic occurrences. SARS-CoV-2 exhibits particular similarities to SARS-CoV which surged into humans in November, 2002 in Foshan, China. This incident reoccurred the following year 2003 in Guangzhou, China (Xu et al., 2004). The emergence of these two SARS-CoV events listed were directly linked to markets that traded animals and the species that were particularly involved were raccoon dogs and civets (Guan et al., 2003). These live animals were also discovered to have been sold in Wuhan markets in 2019 (Xiao et al., 2021). Studies have shown that these animals are susceptible to SARS-CoV-2 infection (Freuling et al., 2020). Studies also revealed that the animal traders in Guangdong province who had no diagnosis of SARS, showed high levels of immunoglobulin G (IgG) to the SARS-CoV virus. The results indicate a 13% overall level and levels >50% for traders that specialized trading civets (Center for Disease Control and Prevention, 2003). Serological surveys that were carried out subsequently revealed approximately 3% positivity rates to SARS-related coronaviruses (SARSr-CoV) in residents of Yunnan province who were known to live in close proximity to bat caves (Wang et al., 2018). This is an indication that rural locations have been regularly exposed to the viruses. Viruses from bats in Yunnan have been documented to share distinct characteristics with SARS-CoV and SARS-CoV-2. There has been considerable difficulty in identifying the pathway that links the emergence of this virus in the human population; this is due to the substantial geographic variation between Yunnan, the location where these bats were specially tested for SARS-CoV and SARS-CoV-2 and the location where the first human case was documented.

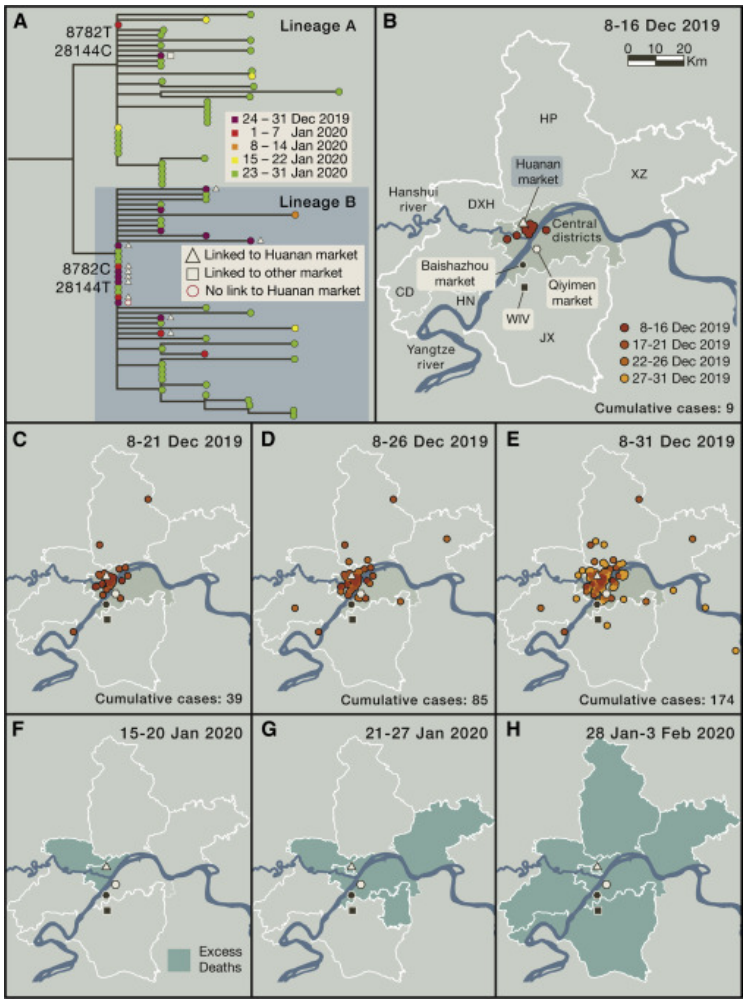

Figure 1. Illustration of epidemiological data on early COVID-19 pandemic in Wuhan (World Health Organization, 2021)

(A) Phylogenetics of SARS-CoV-19 genomes sampled in December 2019-January 2020 from Wuhan. Between A and B lineages, the split has been labeled showing two different coordinates and bases differentiating two nucleotide mutations. (B) The location of markets in districts of Wuhan, also the Wuhan National Biosafety Laboratory. It is the location of the earliest known cases and the coronavirus isolation and culture work of Dr. Shi Zhengli. (C–E) The location of COVID-19 cases recorded from 8-31 of December 2019 in Wuhan, China. (F–H) The map of Wuhan districts highlighting the areas where

the first record of excess deaths due to pneumonia was reported (World Health Organization 2021).

In Wuhan, the Huanan market has been reported to be the major pivot of SARS-CoV-2 infections based on available epidemiological data. Of the three coronavirus disease 2019 (COVID-19) cases that were documented earlier, two were directly linked to the Huanan market, where wild animals were being traded. Approximately 28% of the cases that were reported in December 2019 were also linked to the same location (World Health Organization, 2021). In total, it was documented that 55% of all the cases during December 2019 had contacts or an exposure to either this Huanan or other markets in Wuhan, with the majority of these cases being more prevalent in the first weeks of December (World Health Organization, 2021). Early cases from these locations were further examined and a swarm around Huanan market was observed. In January 2020, cases from these districts were also observed to be the first to manifest excess pneumonia deaths; this is a standard that is less likely to be affected by the potential biases associated with case reporting considering the fact that the case reporting may be indiscriminately subjected to sampling biases reflecting the density and age structure of the population in central Wuhan (Figure 1).

2.1 STRUCTURE OF SARS-CoV-2

Coronaviruses can be classified into four different genera which include: alpha, beta, gamma and delta (Woo et al., 2009). The genome of coronaviruses is a positive-sense single-stranded RNA (+ssRNA).It is the largest known RNA virus. The capsid is formed outside of the genome by the nucleocapsid protein (N). SARS-CoV-2 is the seventh known coronavirus that causes human disease. SARS-CoV-2 was sequenced recently and reports from these studies documented it has a genome size of 29.9 kb (Lu R. et al., 2020). SARS-CoV-2 is made up of four structural proteins: the nucleocapsid protein (N), membrane protein (M), spike protein (S) and the envelope protein (E) (Figure 2). It also contains sixteen non-structural proteins (nsp1−16). Angiotensin-converting enzyme 2 (ACE2) has been reported to be the entry receptor for SARS-CoV-2. These sixteen non-

structural proteins for SARS-CoV-2 have different functions that enhance the pathogenicity of the virus; the main function of the Nsp1 is to initiate the processing and replication of the viral RNA, Nsp2 regulates the survival indication pathway of the host cell. Nsp3 is responsible for the separation of the translated protein. Nsp4 modifies ER membranes. Nsp5 participates in the process of polyprotein during replication. Nsp6 is suspected to be transmembrane domain. The combination of nsp12 and template-primer is significantly increased by presence of nsp7 and nsp8. ssRNA-binding protein is controlled by Nsp9. Nsp10 is used for the cap methylation of viral mRNAs. Nsp12 contains the RNA-dependent RNA polymerase (RdRp), which is critical for replication/transcription composition of coronavirus. Nsp13 binds with ATP and the zinc-binding domain in nsp13 participates in the process of replication and transcription of the virus. Nsp14 is a domain that proofreading exoribonuclease. Mn(2+)- endoribonuclease activity is dependent on Nsp15. Nsp16 is a 2'-O-ribose methyltransferase (Naqvi et al., 2021).

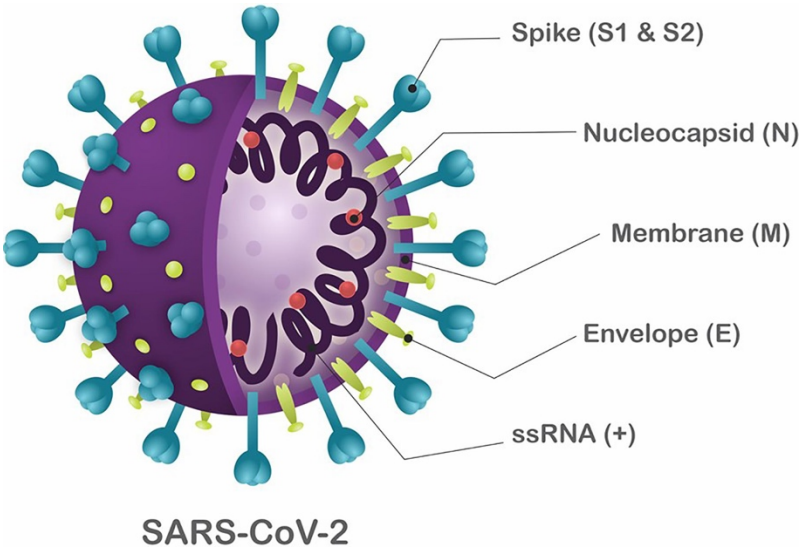

Figure 2. Schematic structure of SARS-CoV-2 (Fehr and Perlman, 2015).

2.1.1 SPIKE GLYCOPROTEIN

Spike glycoprotein (S protein) facilitates the entry of coronaviruses into the host cells (Li et al., 2003; Li et al., 2005; Li, 2016). Upon binding, the transmembrane forms a homotrimers that protrudes to the viral surface. This spike glycoprotein is fundamental for the coronavirus entry into the host cells as it has a very critical role in the process of receptor recognition and the fusion of the virus cell membrane. These characteristics also make it a suitable as an antiviral target. There are two functional subunits of the spike protein; which are S1 and S2 subunits (Figure 3). The S1 subunit is made up of a receptor binding domain (RBD). The function of this domain is to recognize and bind to the host receptor ACE2 on host cell, while the S2 subunit forms a six helical bundle via the two-heptad repeat domain which enables it to initiate a viral cell membrane fusion (Walls et al., 2020; Wrapp et al., 2020).

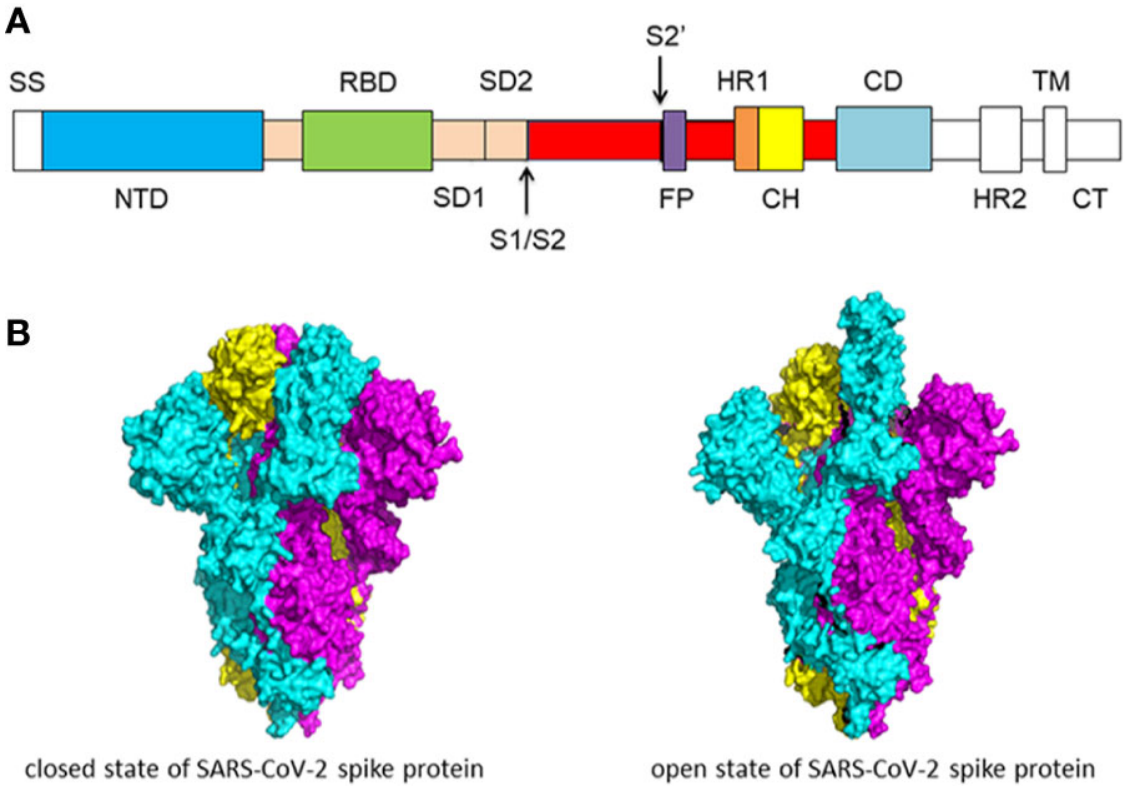

Figure 3. (A) Schematic representation of SARS-CoV-2 spike protein structure (B) Cryo-electron micrograph of SARS-CoV-2 spike protein structure (Walls et al., 2020)

The structure of SARS-CoV-2 exhibit very distinct similarities with the SARS-CoV S protein. S1 and S2 subunits are known to remain non-covalently bound in the prefusion conformation. Special domains are utilized by the S1 subunits which facilitates the recognition of different entry receptors. SARS-CoV and SARS-CoV-2 recognize the receptor angiotensin-converting enzyme 2 (ACE2) on host cells before entry via the RBD (Walls et al., 2020).

2.2 EVOLUTION OF SARS-CoV-2 VARIANTS

In the process of genome replication of viruses, changes in the genetic codes (genetic mutation) occurs leading to the continuous evolution of viruses such as SARS-CoV-2. A virus lineage is group of genetically closely related group of virus lineage that are derived from a common ancestor; while a virus variant has one or more mutations that differentiate it from other variants of a known virus.

The genetic diversity of all coronaviruses is made possible by a large genome (26.4-31.7kb) (Woo et al., 2010). Recent estimates indicate that SARS-CoV-2 evolutionary rate has undergone a substantial increase to 6.6/1,000 nucleotides/year. Accordingly, multiple reports show emergence of SARS-CoV-2 variants and clades. Since the emergence of SARS-CoV-2, over 20,000 viral mutations including several insertions/deletions, have been reported within the viral genome (Wu et al., 2021). The variants of SARS-CoV-2 have been grouped into different clades (Table 1); there are several nomenclatures for the SARS-CoV-2 clades, each health organization uses its own identifiers for the different variants. According to the Global Initiative on Sharing Avian Influenza Data (GISAID) the variants have been separated into nine different clades which include; S, O, L, V, G, GH, GR, GV, and GRY. The S and L clades were documented to have been around at the beginning of the global pandemic. The S clade was prevalent at the initial stage whilst the L clade divided into G and V, further split into GR, GH, and GV. GR further split into GRY in August 2020. The letters have been used based on the mutations that caused the branching of these variants (GISAID, 2021).

A subset of these variants have been reported to increase viral transmission, such as Alpha B.1.1.7 and Delta B.1.617.2, while some variants have been reported to escape humoral immunity (Beta B.1.351 and Gamma P.1). In particular, the spike glycoprotein has been characterized by the faster accumulation of mutations due to its critical role in mediating viral infectivity and antigenicity. Mutations in the spike glycoprotein have raised global concerns for their link with enhanced transmissibility, greater disease severity, risk of re-infection, potential impact in diagnostics, and decreased efficacy of vaccines (Forni et al., 2021).

A variant of concern (VOC) is a variant for which an evidence of increased transmissibility, disease severity, and significant reduction in neutralization by antibodies generated during previous infection or vaccination, reduced effectiveness of treatments or vaccines, or diagnostic detection failures exist. Depending on the epidemiological characteristics and patterns of spike mutations, four variants have been classified as VOCs by the WHO, U.S. Centers for Disease Control and Prevention (CDC), and European Centre for Disease Prevention and Control (ECDC). These variants, including B.1.1.7, B.1.351, P.1, and B.1.617.2 (recently renamed by WHO as Alpha, Beta, Gamma, and Delta, respectively) were first identified in the United Kingdom, South Africa, Brazil, and India, respectively, and are currently dominant strains circulating in multiple other countries. These strains have been named using the PANGOLIN lineage terminology.

The most recently discovered SARS-CoV-2 variant was designated by the WHO in November, 2021, the variant is B.1.1.529 named Omicron. The decision was based on the evidence that were presented to the Technical Advisory Group on SARS-CoV-2 Virus Evolution (TAG-VE) stating that the variant possesses numerous mutations that has the ability to impact its transmissibility and the severity of illness caused. This variant was first discovered by researcher in South Africa in November 2021.

Table 1. SARSCoV-2 variants and their PANGOLIN lineage equivalent using Greek lettering system (World Health Organization, 2021)

Variants of Concern			
Name	**Lineage**	**Location of emergence**	**Date of detection in the human population**
Alpha	B.1.1.7	Britain	December 2020
Beta	B.1.351	South Africa	December 2020
Gamma	P.1	Brazil	January 2021
Delta	B.1.617.2	India	December 2020
Omicron*	B.1.1529	Multiple Countries	November 2021
Variants of Interest			
Name	**Lineage**	**Location of emergence**	**Date of emergence**
Epsilon	B.1.427, B.1.429	California	February 2021
Zeta	P.2	Brazil	February 2021
Eta	B.1.525	New York	February 2021
Theta	P.3	Philippines	June 2021
Iota	B.1526	New York	February 2021
Kappa	B.1617.1	India	May 2021

2.3 SARS-CoV-2 TRANSMISSION

SARS-CoV-2 is a highly infectious pathogen that causes a severe respiratory infection named COVID-19. It has certain pneumotropic features which can be confirmed based on the viral uptake and its replication in epithelial cells. This virus is transmitted from person-to-person mainly through respiratory secretions such as aerosol droplets that are produced when an infected person coughs, sneezes, and talks (Figure 4). Patients are known to suffer from various symptoms such as cough, fever, shortness of breath, gastrointestinal symptoms, muscle ache and headache. Respiratory fluids carrying infectious virus are the primary mode by which the virus is transmitted. Exposure to the

virus occurs in three principal ways: (1) inhalation of respiratory droplets and aerosols, (2) deposition of respiratory droplets on mucous membranes in the mouth, nose, or eye by direct splashes and sprays, and (3) touching mucous membranes with hands that have been contaminated either directly by virus-containing respiratory fluids or indirectly by touching surfaces covered with viral particles.

Respiratory fluids are released during exhalation (e.g., quiet breathing, speaking, singing, exercise, coughing, sneezing) in the form of droplets across a range from different sizes (Echternach et al., 2020). These droplets carry virus and transmit infection. Large droplets settle out of the air rapidly, within seconds to minutes while smaller, very fine droplets and aerosol particles remain suspended in the air for minutes to hours. Besides airborne transmission, fecal–oral human-to-human transmission events have also been reported (Figure 3).

There are three main ways by which individuals can be exposed to infectious respiratory fluids of SARS-CoV-2. These three routes can exist independently and can overlap with one another.

1. Through inhalation of droplets and aerosols that contain infectious virus. A higher concentration of the virus is contained within three to six feet of an infectious source which increases the risk of infection.
2. Depositing virus carried in exhaled droplets and particles onto exposed mucous membranes (i.e., “splashes and sprays”, such as being coughed or sneezed on). The risk of infection is higher at a closer distance.
3. Touching mucous membranes with hands that have come in contact with infectious exhaled respiratory fluids containing virus or from touching inanimate objects and surfaces that have been contaminated with virus.

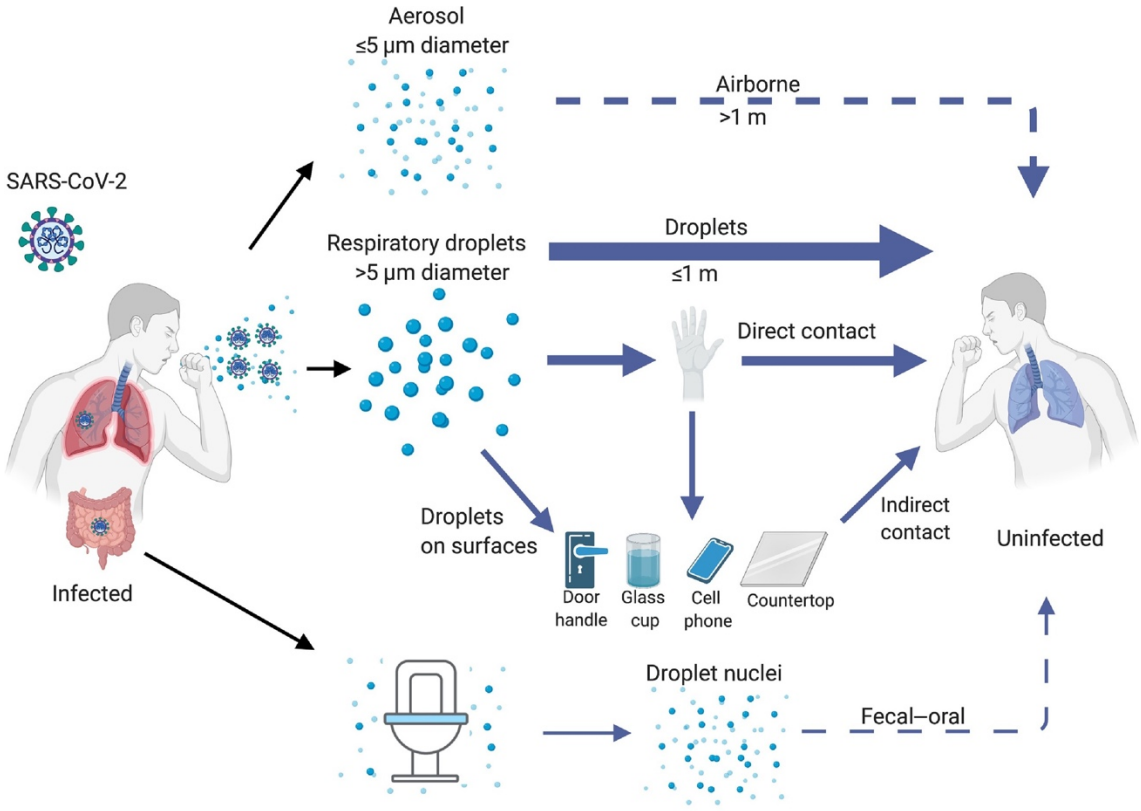

Figure 4. Proposed transmission routes for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Mukhra et al., 2020).

Once infectious droplets and particles are exhaled, they move outward from the source. The risk for infection decreases with increasing distance from the source and increasing time after exhalation. Two principal processes determine the amount of virus to which a person is exposed in the air or by touching a surface contaminated by virus:

1. Decreasing concentration of virus in the air: as larger and heavier respiratory droplets containing virus fall to the ground or other surfaces under the force of gravity and the very fine droplets and aerosol particles that remain in the airstream progressively mix with, and become diluted within, the growing volume and streams of air they encounter. This mixing is not necessarily uniform and can be influenced by thermal layering and initial jetting of exhalations.

2. Progressive loss of viral viability and infectiousness: this is influenced by environmental factors and such as temperature, sunlight and humidity.

With increasing distance from the source, the risk inhalation likewise increases. Although infections through inhalation at distances greater than six feet from an infectious source are less likely than at closer distances, the phenomenon has been repeatedly documented under certain preventable circumstances (Bae et al., 2020). These transmission events have involved the presence of an infectious person exhaling virus indoors for an extended time (more than 15 minutes and in some cases hours) leading to virus concentrations in the air space sufficient to transmit infections to people more than 6 feet away, and in some cases to people who have passed through that space soon after the infectious person left. Per published reports, factors that increase the risk of SARS-CoV-2 infection under these circumstances include:

- Enclosed spaces with inadequate ventilation causing the concentration of exhaled respiratory fluids, especially smaller droplets and aerosols can build-up in the air space.
- Increased exhalation of respiratory fluids if the infectious person is engaged in physical exertion or raises their voice (e.g., exercising, shouting, and singing).
- Prolonged exposure to these conditions, typically more than 15 minutes.

2.4 PATHOGENESIS OF SARS-CoV-2

There are three major phases of SARS-CoV-2 pathogenesis which are correlated to the different clinical stages of the disease. The first stage, which is usually an asymptomatic stage begins with the inhalation where the viral agent gains entry into the host cells and binds to the epithelial cells of the nasal cavity. The main receptor for the SARS-CoV-2 virus is ACE2 (Wan et all., 2020; Hoffman et al., 2020). The virus begins to propagate in cells, however, there is a minimal level of innate immune response. The virus can be detected in specimens from nasal swabs during this stage. Whilst the viral load at this stage can be observed to be low, these individuals are still infectious. RT-PCR

test and the corresponding results can be used to determine the level of the viral RNA which is useful to predict the viral load and the subsequent infectivity and clinical course.

The second phase is characterized by the clinical manifestation, a more intense innate immune response is triggered as the virus propagates and migrates down the respiratory tract along the conducting airways. Nasal swabs or sputum specimens obtained at this stage should show the presence of SARS-CoV-2 as well as early indications of the innate immune response. The level of certain innate response cytokines such as CXCL10 can be used to predict subsequent clinical course (Tang et al., 2018). Epithelial cells that have been infected by the virus are a major source of beta and lambda interferons (Hancock et al., 2018).

In most cases, the disease will be mild and restricted to the upper and conducting airways of infected patients (Wu and McGoogan 2020). Symptomatic therapy and home care is recommended for these individuals. However, in very few cases, the disease progresses to stage 3 in infected patients resulting in the development of pulmonary infiltrate or a more severe disease. The fatality rate at this stage is approximately 2%, but this varies with age (Wu and McGoogan 2020). In this stage, the virus spreads to the gas exchange units of the lung, infecting alveolar type II cells. This is observed pathologically by a diffused damage in the alveolar with the hyaline membranes being filled with fibrin (Gu and Korteweg, 2007). The healing of this damage may result in more severe scarring and fibrosis than other forms of acute respiratory distress syndrome (ARDS). Recovery requires a vigorous innate and acquired immune response and epithelial regeneration. Due to the weakened immune response and reduced ability to repair the damaged epithelium of the elderly, they have become particularly at risk The virus also has a higher tendency to spread to the gas exchange units of the lung in elderly individuals as a result of their reduced mucociliary clearance (Ho et al., 2001).

2.5 HOST IMMUNE RESPONSE TO SARS-CoV-2

Understanding the host immune response to SARS-CoV-2 infection is of utmost importance. Following infection with SARS-CoV-2 or vaccination, it is the adaptive immune response that ideally delivers long-term protection (Figure 5). The adaptive immune response primarily comprises memory B cells that produce different classes of antibody to neutralize the virus or virus-infected cells, and memory T cells that support antibody production and also have a direct role in killing virus-infected cells. While there is evidence of both memory B cell and T cell immune responses in individuals infected with SARS-CoV-2 as well as in vaccinated persons, a clear sum for protective immunity is yet to be defined (Huang et al., 2020 and Hellerstein 2020). The foremost current indication for protection against re-infection for previously infected individuals or breakthrough infection in vaccinated individuals is the presence of neutralizing antibodies against SARS-CoV-2. S1 domain of the SARS-CoV-2 spike protein includes the receptor-binding domain (RBD), and antibodies targeting this antigen critically impair virus cell entry (Letko et al., 2020). Few studies have shown that neutralization ability of polyclonal antibodies in serum tends to correlate positively with anti-spike IgG or anti-RBD IgG (Post et al., 2020).

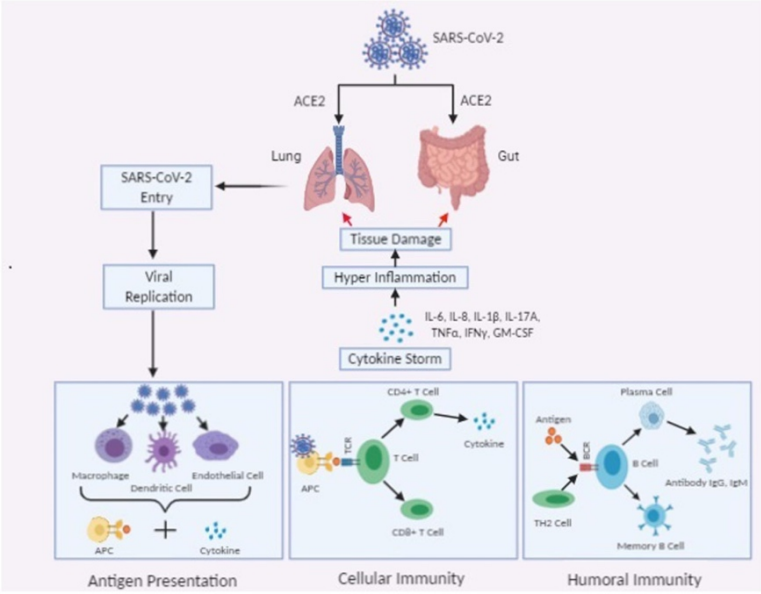

Figure 5. Mechanism of the immunopathogenesis of SARS-CoV-2 infection (Zhou Z. et al. 2020).

2.5.1 IMMUNE RESPONSE FOLLOWING NATURAL INFECTION

Results obtained from a cohort study describing the virus-specific serum indicated that that immunoglobulin M (IgM) is consistently detected before IgG, peaking between weeks two and five and declining later on. Furthermore, the assessment of immune responses induced by vaccination has majorly been focused on the development of antibodies targeting the S1 domain of the SARS-CoV-2 spike protein. One outstanding benefit of the vaccine regimens is that the anti-S IgG titres are higher than for natural infection, with the sera from vaccinated individuals showing greater neutralization capacity against homologous SARS-CoV-2 viruses *in vitro* (Grigoryan and Pulendran 2020).

Susceptibility to the progression of infection as well as a major determinant of recovery of infected individuals is dependent on the host immune response to COVID-19. This is mobilized by coordinated B and T cell responses (Grifoni et al., 2020). It is important to note that for the continuous development of successful therapeutic agents and against COVID-19, more data regarding the longevity of immunity to SARS-CoV-2 is required. The present knowledge and understanding of SARS-CoV-2 immunity is mainly based on previous studies and experiences with other coronaviruses and viral infections.

2.5.2 INNATE IMMUNE RESPONSE

Innate immunity is known as the first line of defense for the immune system and has an important function of cell-autonomous response for virus infected cells thatis influenced by receptors and co-receptors of viral entry (Hoffman et al., 2020; Wu et al., 2020). Studies have shown that the surface receptor enzyme for SARS-CoV-2, the ACE2 was the cellular entry point for SARS-CoV-2 (Hoffman et al., 2020). The innate response of SARS-CoV-2 is triggered by pattern recognition receptors (PRRs).This is particularly due to the fact that SARS-CoV-2 is an RNA virus, which enables detection of the virus (Dixit and Kagan 2020) and results in the downwards signaling cascade, leading to the secretion of cytokines such as type I/III interferons (IFNs), tumor necrosis factor alpha

(TNF-a), interleukin-1 (IL-1), and IL-6 among other cytokines. The release of the cytokines prompts antiviral activity in the host cells, which subsequently elicits adaptive immune responses. It is also important to note that if released early as well as being properly localized, IFN-1 can be an effective control for SARS-CoV-2 infection (Kindler and Thiel 2020).

2.5.3 DURATION OF PROTECTIVE IMMUNITY

Although virus-specific B cell and T cell responses can be detected shortly into the recovery from infection or vaccination, they wane over time, with decreasing numbers of circulating virus-specific memory T cells, memory B cells, and serum antibodies. Consequently, protection against outcomes such as infection, transmission risk, or severe disease may also wane over time. To fully understand and examine waning immunity, both the quantitative reduction in immune readouts and the qualitative or functional change over time should be carefully evaluated. It is also important to consider the interdependency of readouts assessing T cells, B cells, or antibodies. In a long-term follow-up study of 25 individuals infected with SARS-CoV-2, virus-specific memory B cells were identified in the early stages of convalescence. While serum antibodies peaked 20 days post-infection before waning, virus-specific memory B cells persisted for over 242 days post-symptom onset (Hartley et al., 2020). The development of memory T cells directed at non-surface SARS-CoV-2 proteins following infection or vaccination may offer a route to durable immunity where virus evolution leads to spike protein mutations that escape pre-existing neutralizing antibodies (Tarke et al., 2021). This will occur either by offering more efficient support to activated naïve B cells responding to the altered spike protein (memory CD4 T cells), or through direct lysis of SARS-CoV-2 infected cells (CD8 T cells) (Dan et al., 2021; Tarke et al., 2021).

The vast majority of SARS-CoV-2-infected individuals seroconvert following SARS-CoV-2 infection. Results from studies showed that >90% patients develop IgG seropositivity and neutralizing antibodies following primary infection, ranging between 91 and 99% in large studies (Post et al., 2020; Dan et al., 2021). A scoping review performed by the Irish Health Information and Quality Authority (HIQA) to evaluate the

long-term duration of immune responses following SARS-CoV-2 infection identified five studies that investigated immune responses at ≥6 months post-infection, including two studies at ≥8 months post-infection. In general, studies reported a waning of antibody responses in the late convalescent period (3-6 months post-infection). However, T-cell and memory B-cell responses were still present, and in many cases increased, up to eight months post-infection in all study participants (HIQA,2021).

Follow-up periods for previously infected and vaccinated individuals are not yet sufficiently long enough to be able to draw conclusions on the duration of protection against infection beyond 6 months for those vaccinated or beyond 12 months post-natural infection.

2.6 CLINICAL MANIFESTATIONS OF COVID-19

The most common symptom indicated by patients with mild or moderate disease are headache, loss of smell, nasal obstruction, cough, asthenia, myalgia, rhinorrhea, gustatory dysfunction, sore throat and fever (Lechien et al., 2020). Olfactory and gustatory dysfunctions have been identified as common symptoms (Tong et al., 2020). Additionally, altered taste sensation was found among of COVID-19 patients (Aziz et al., 2020). Children were most commonly reported to show symptoms of fever and cough. Other symptoms include gastrointestinal symptoms, sore throat/pharyngitis, shortness of breath, myalgia, rhinorrhoea/nasal congestion and headache with varying prevalence among different studies (Mantovani et al., 2020; Raba et al., 2020; Patel 2020).

Although most cases of COVID-19 are mild or moderate and do not require hospitalization or advanced medical care, severe cases have been recorded. The severity of COVID-19 varies amongst different age groups. A strong gradient of severity was recorded in the elderly as well as people with underlying health issues. Patients with COVID-19 often report persisting symptoms or develop new symptoms after recovery from an acute infection (Nalbandian et al., 2021). At least one symptom has been reported in up to 80% of individuals beyond two weeks after a confirmed COVID-19 diagnosis (Lopez-Leon et al., 2021).

Symptoms that persist beyond three weeks from the onset of symptoms are defined as ‘post-acute COVID-19’ and persistence of symptoms beyond 12 weeks is defined as ‘chronic COVID-19’ (Greenhaigh et al., 2020). The term ‘sub-acute or ongoing symptomatic COVID-19’ is proposed when symptoms persist for four to twelve weeks after acute COVID-19. Prolonged symptoms may be due to delayed recovery from the acute infection, including post-intensive care unit (ICU) syndrome, ongoing infection, organ damage and may also be due to complications (such as myocardial injury, pulmonary embolism, lung fibrosis and stroke), multisystem inflammatory syndrome (MIS), and post-viral fatigue syndrome (National Institute for Health Research 2020; Mahase 2020), or a combination of all of the above.

2.7 COVID-19 DIAGNOSTIC TESTS

Optimal specimens for detection of current infection with SARS-CoV-2 are collected from the upper respiratory tract (nasal wash, nasopharyngeal aspirate, nasopharyngeal swab, oropharyngeal swab). However, if the patient is hospitalized or under intensive care, specimens can also be collected from the lower respiratory tract (bronchoalveolar lavage (BAL) and endotracheal aspirate). There are three major approaches that can be used in the clinical diagnosis of COVID-19. These include; epidemiological history, clinical symptoms and laboratory examinations (del Rio, Malami 2020); Gorbalenya, 2020; Corman et al., 2018). Laboratory test are critical for the confirmation of diagnosis even after an infection has been considered to be diagnosed clinically by case history and symptoms. The diagnostic test to be used should be evaluated according to the kinetics of SARS-CoV-2 markers during infection (Figure 6). The strategies used for the diagnosis of COVID-19 are described below.

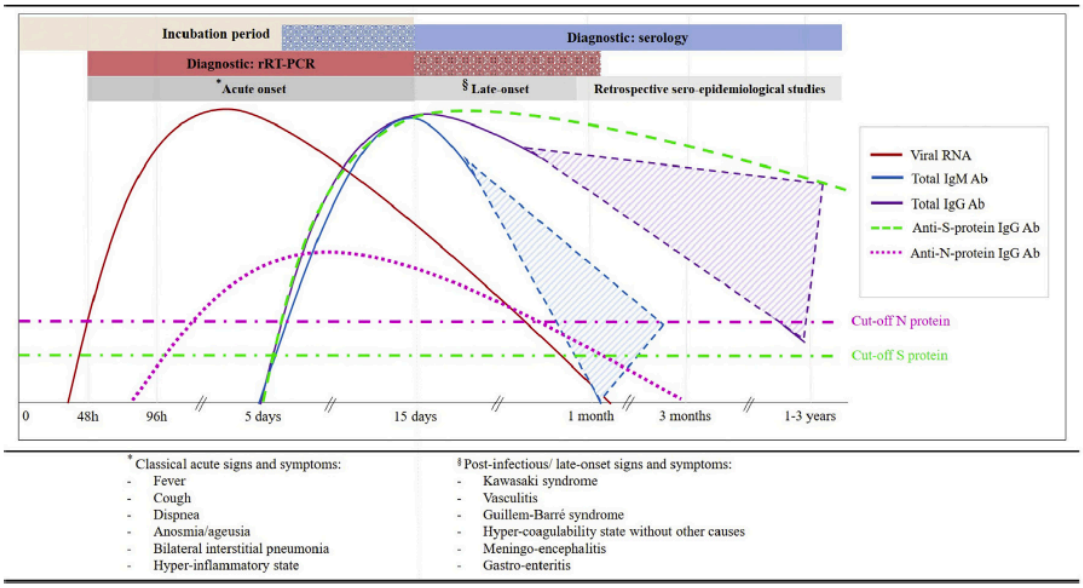

Figure 6: Kinetics of SARS-CoV-2 markers during infection and laboratory diagnosis (Zheng and Song 2020).

2.7.1 ANTIBODY TESTING FOR COVID-19

Antibody test are used for the detection of antibodies from a prior or recent COVID-19 infection. Positive antibody results can also occur from vaccination. For optimal results, the specimens for antibody testing should be acute and convalescent (two or four weeks after acute phase of infection, serum or plasma) (Figure 7). Serological testing should not be used to detect current infection with SARS-CoV-2 in clinical care. Immunoglobulin M (IgM) antibodies can be detected early in an infection, while immunoglobulin G (IgG) antibodies are more likely to develop later in the infection, usually 14 days after the onset of symptoms. A positive antibody results is an indication of exposure to the virus and immunity. However, the duration of this immunity is still unclear.

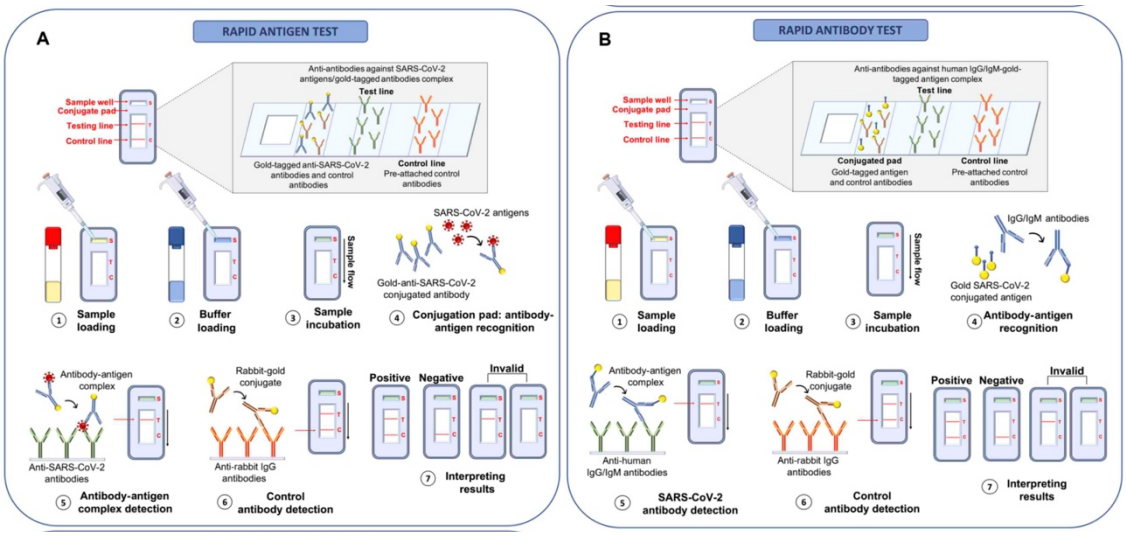

Figure 7. Rapid antigen and rapid antibody tests. (A) Workflow of rapid antigen test for the detection of SARS-CoV-2 viral antigens through lateral flow immunoassay (B) Workflow of rapid antibody test for the rapid detection of human IgG, IgA, or IgM antibodies against SARS-CoV-2 antigens via lateral flow immunoassay (Lee et al., 2019; Diao et al., 2021)

2.7.2 PCR-BASED METHODS

PCR test is considered the gold standard of SARS-CoV-2 detection. It is used for the detection of RNA that is specific to the virus and is capable of detecting the virus within days of infection, even in asymptomatic patients and produces results within 24 hours. Real-time RT-PCR method was developed for the specific detection of genetic materials in real time using special markers such as fluorescent dyes by creating multiple fragments of genetic material through a process known as amplification. In order for the COVID-19 virus to be detected using RT-PCR, the RNA virus needs to be converted to DNA by reverse transcriptase as only DNA can be amplified (Figure 8). RT-PCR is used mainly due to its high sensitivity and ability to produce reliable results in hours. This assay targets the SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) gene, as well as the E and N genes. The Ct value of an RT-PCR assay is the number of cycles at which fluorescence of the PCR product is detectable over and above the background signal. The

Ct value is inversely correlated with viral load i. e. a higher number of cycles required shows that the virus was undetected at lower cycles, which is an indication of a low viral load. The accepted benchmark for COVID-19 CT value ranges between 35-40.

The significance of the CT value in RT-PCR tests is that it serves as a measure for viral load and a parameter for determination of transmissibility and its influence on severity of infection is still being studied.

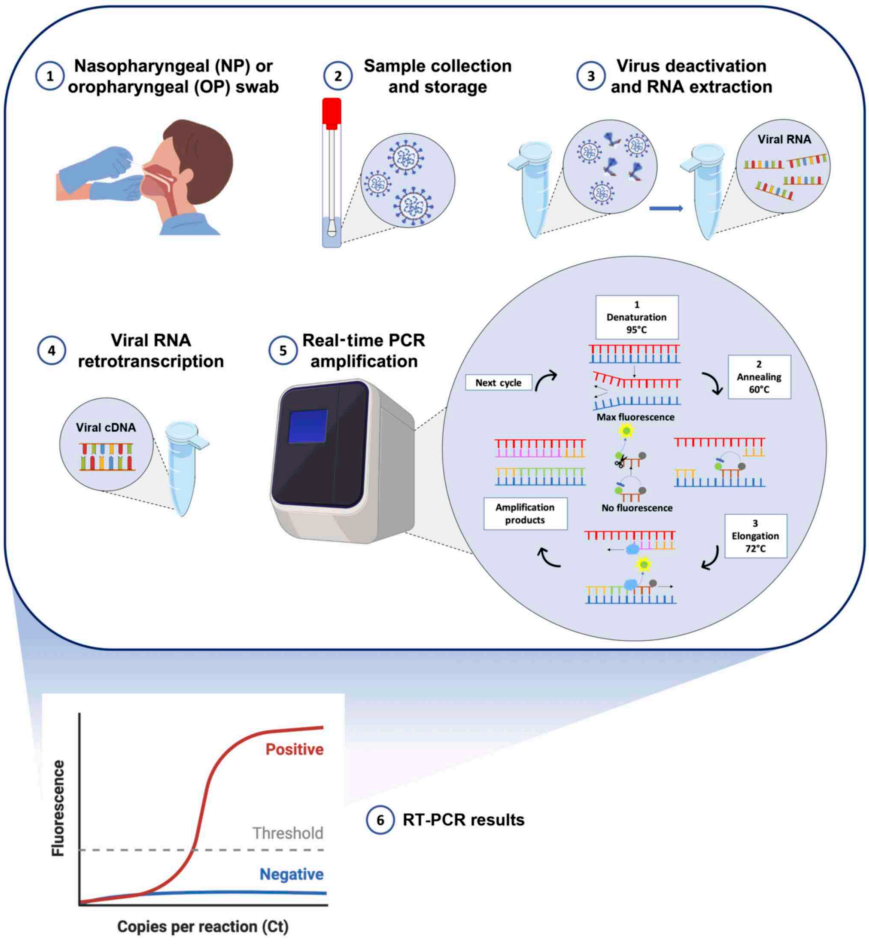

Figure 8. Schematic workflow of RT-PCR-based diagnostic method (Fomgaard and Rosenstierne, 2020).

2.7.3 DETECTION OF VIRAL ANTIGENS

Viral antigen test for COVID-19 is used for the rapid detection of an active infection primarily by detecting the nucleocapsid protein antigen of SARS-CoV-2 virus using nasopharyngeal or similar specimen. The process begins with the sample collection and takes approximately 15 minutes (Figure 7). However, these tests are generally less sensitive than molecular test. This lower sensitivity increases the possibility of a false negative result. Antigen tests are more likely to detect a true positive at the early stage of the infection due to the high viral carriage at the early stage. This rapid antigen test provides qualitative detection of IgG and/or IgM in patients' samples. It is based on the principle of lateral flow immunoassay chromatography, the separation of components of a mixture through a medium using capillary force and the specific binding of an antibody to its antigen. A positive antigen test result is indicated by the binding of anti-SARS-CoV-2 antibodies present to antigens present in the conjugation pas of cassette and this complex formed with migrate to the membrane-bound anti-human IgG and IgM.

2.7.4 CHEST X-RAY RADIOGRAPHS IN PATIENTS WITH COVID-19

Chest X-rays are used for the diagnosis and follow up of patients with COVID-19 pneumonia. In addition to certain clinical symptoms, chest X-ray can be used to diagnose a person with suspected or confirmed COVID-19 disease. Bilateral pulmonary parenchymal ground-glass, consolidative pulmonary opacities are some of the most common findings with chest X-rays (Figure 9). In severe cases, infection with SARS-CoV-2 may develop into radiographic changes known as "white livers."

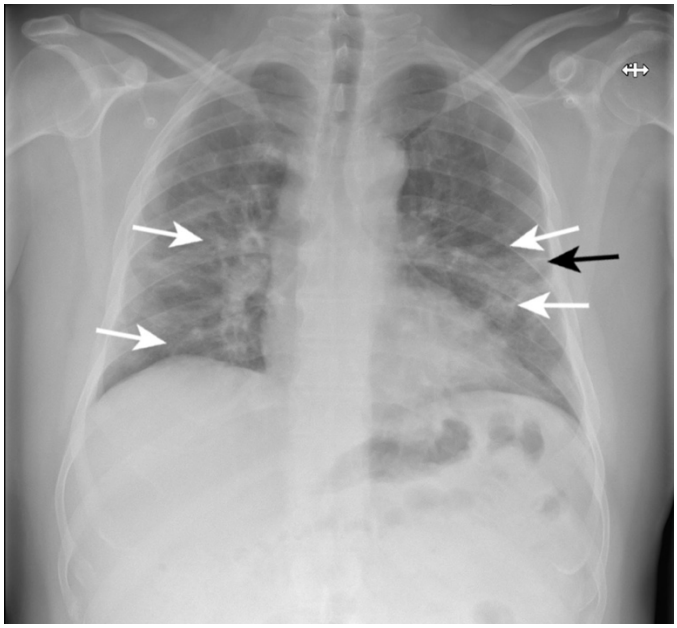

Figure 9. Posterior-anterior chest radiograph of a patient with COVID-19 pneumonia showing ground glass opacity in mid and lower zones of the lungs. Linear opacity observed in the periphery of the left mid zone (black arrow) and peripheral glass opacity (white arrows) with lung marking preservation (Hansell et al., 2008).

2.8 TREATMENT OF COVID-19

The treatment for COVID-19 depends on the severity of the infection. For milder cases, isolation, resting at home and use of medication to reduce the fever is often sufficient. More severe cases may require hospitalization and treatments that include support care, assisted ventilation and supplemented oxygen. Due to the high transmissibility of SARS-CoV-2, the need for the development of effective treatment agents is critical and time sensitive. In this regard, a variety of drugs, procedures and devices are being investigated, while some have received approval for use from the United States Food and Drugs Administration (FDA).

2.8.1 ANTIVIRAL DRUGS FOR COVID-19 TREATMENT

Currently, some antiviral agents have been approved by the FDA for the treatment of COVID-19. The expanded access use (EA) and emergency use authorization (EUA) programs created by the FDA allowed clinicians to gain access to investigational therapy. Antiviral agents that were granted approval by the FDA include:

REMDESIVIR

The drug remdesivir was the first approved by the FDA for the treatment of SARS-CoV-2 infection and is indicated for the treatment of COVID-19 disease. This drug has been authorized for in hospitalized adults and child aged 12 year and older who weigh at least 40 kg. Remdesivir is a broad-spectrum antiviral and a nucleotide analog prodrug. It first received a US FDA issue EUA in May, 2020 and allowed by the FDA for prescription in severe COVID-19 hospitalized adults and children prior its approval (Food and Drug Administration, 2020).

FAVIPIRAVIR

Favipiravir (Avigan, Reeqonus; Appili Therapeutics) is an oral antiviral that disrupts viral replication by selectively inhibiting RNA polymerase. An adaptive, multicenter, open label, randomized, phase 2/3 clinical trial of favipiravir compared with standard of care I hospitalized patients with moderate COVID-19 was conducted in Russia. Both dosing regimens of favipiravir demonstrated similar virologic response. Viral clearance on day 5 was achieved in 25/40 (62.5%) patients on in the favipiravir group compared with 6/20 (30%) patients in the standard care group ($p = 0.018$). Viral clearance on day 10 was achieved in 37/40 (92.5%) patients taking favipiravir compared with 16/20 (80%) in the standard care group ($p = 0.155$) (Ivashchenko et al., 2020).

MOLNUPIRAVIR

An EUA for molnupiravir was requested in October 2021. This drug was narrowly voted by the FDA's Antimicrobial Drugs Advisory Committee recommending the FDA's authorization. This is based on data from November 30, 2021.

Molnupiravir (MK-4482 [previously EIDD-2801]; Merck and Ridgeback Biotherapeutics) is an oral antiviral agent that is a prodrug of the nucleoside derivative N4-hydroxycytidine. It elicits antiviral effects by introducing copying errors during viral RNA replication of the SARS-CoV-2 virus.

The phase 3 MOVe-OUT study (n=1433) has shown that molnupiravir reduced risk of hospitalization or death from 9.7% (68 of 699) in the placebo group to 6.8% (48 of 709) in the molnupiravir group for an absolute risk reduction of 3% ($p = 0.02$) and a relative risk reduction of 30%. Nine deaths were reported in the placebo group and 1 in the molnupiravir group (Fischer et al., 2021). Molnupiravir is also being evaluated in a phase 3 trials for postexposure prophylaxis for individuals residing in the same household with someone who tests positive for SARS-CoV-2 in the phase 3 MOVE-AHEAD trial (Study MK-4482, 2021).

2.8.2 ANTI-INFLAMMATORY DRUGS

Some of the anti-inflammatory drugs recommended for use include: glucocorticoids and methylprednisolone. Based on the severity of systematic response, degree of shortness of breath, presence or absence of ARDS, and the chest imaging results observed in some patients, glucocorticoids received approval from the FDA to be used as an anti-inflammatory drug of choice. Furthermore, the use of immunomodulatory agents, such as tocilizumab (IL-6 inhibitor) and anakinra (IL-1 receptor antagonist) can be of tremendous help as the cytokine storm has been observed to be more common in severe cases of COVID-19 (Zhang et al., 2020).

2.8.3 CONVALESCENT PLASMA THERAPY

This is a method of antibody therapy, which is solely based on the ability of the immune system of an infected person to produce antibodies to fight the virus after they have been infected and recovered (convalesce). This method of treatment received an EUA from the FDA in August, 2020 for plasma antibodies.

2.9 VACCINES AGAINST SARS-CoV-2

Vaccines are the best way to train our immune system to recognize viruses, or pieces of viruses called antigens. Our immune system creates antibodies and other defenses to protect us. When a vaccinated person is exposed to SARS-CoV-2, their immune system will recognize the viral antigens. There are various different types of vaccines as described below.

2.9.1 TYPES OF VACCINE PLATFORMS

COVID-19 vaccines are being developed using several different platforms (Krammer, 2020). Some of these are traditional approaches, such as inactivated virus or live attenuated viruses, which have been used for inactivated influenza vaccines and measles vaccine, respectively. Other approaches employ newer platforms, such as recombinant proteins (used for human papillomavirus vaccines) and vectors (used for Ebola vaccines). Some platforms, such as RNA and DNA vaccines, had never been employed in a licensed vaccine. General descriptions of the different platforms used for COVID-19 vaccines are presented here. There are two main categories of COVID-19 Vaccines: component viral vaccines and whole virus vaccines.

2.9.1.1 Component Viral Vaccines

- **Protein Subunit**: Contains isolated and purified viral proteins
- **Virus-like Particles (VLP)**: Contains viral proteins that mimic the structure of the virus, but no genetic material
- **DNA-based** and **RNA-based**: Contains viral genetic material (such as mRNA) which provides the instructions for making viral proteins
- **Non-Replicated Viral Vector**: Contains viral genetic material packaged inside another harmless virus that cannot copy itself
- **Replicating Viral Vector**: Contains viral genetic material packaged inside another harmless virus that can copy itself

2.9.1.2 Whole Virus Vaccines

- **Inactivated**: Contains copies of the virus that have been killed (inactivated)
- **Live-Attenuated**: Contains copies of the virus that have been weakened (attenuated)

● **Inactivated vaccines** – Inactivated vaccines are produced by growing SARS-CoV-2 in cell culture then by chemically inactivating the virus (Plotkin et al., 2017 and Gomez & Robinson 2018). The inactivated virus is often combined with alum or another adjuvant in the vaccine to stimulate an immune response. Inactivated vaccines are typically administered intramuscularly. They require a biosafety level 3 facility for production. Immune responses to a SARS-CoV-2 inactivated vaccine would target not only the spike protein but also other components of the virus.

● **Live attenuated vaccines** – Live attenuated vaccines are produced by developing genetically weakened versions of the wild-type virus; these weakened viruses replicate in the recipient to generate an immune response but do not cause disease (Plotkin et al., 2017 and Gomez & Robinson 2018). Attenuation can be achieved by modifying the virus genetically or by growing it in adverse conditions so that virulence is lost but immunogenicity is maintained. A live attenuated COVID-19

vaccine would hopefully stimulate both humoral and cellular immunity to multiple components of the whole attenuated virus. Another advantage of live vaccines is that they can be administered intranasally, as with the live attenuated influenza vaccine which might induce mucosal immune responses at the site of viral entry in the upper respiratory tract. However, safety concerns with live attenuated vaccines include reversion to or recombination with the wild-type virus.

● **Recombinant protein vaccines** – Recombinant protein vaccines are composed of viral proteins that have been expressed in one of various systems, including insect and mammalian cells, yeast cells, and plants. These vaccines are typically administered intramuscularly. They do not require replication of the live virus, which facilitates production, although production yields depend on the ability to express the spike protein, which is variable. Recombinant COVID-19 vaccines in development include recombinant spike protein vaccines, recombinant receptor-binding domain vaccines, and virus-like particle (VLP) vaccines (World Health Organization 2020).

● **Vector vaccines**

• **Replication-incompetent vector vaccines**– In this platform, a different vector virus that has been engineered to not replicate *in vivo* and to express the viral protein that is the intended immune target is used. Many replication-incompetent vector vaccine candidates use adenovirus vectors (World Health Organization, 2020). A potential disadvantage of the vector vaccines is that pre-existing immunity to the vector can attenuate immunogenicity of the vaccines (Zhu et al., 2020). This can be avoided by using viral vectors that are rare in humans, vectors derived from animal viruses, such as a chimpanzee adenovirus, or vectors that do not induce self-immunity, such as adeno-associated viruses (AAV). Most SARS-CoV-2 replication-incompetent vector vaccines are administered intramuscularly and are devised to express the spike protein, with a resultant host immune response to that protein.

• **Replication-competent vector vaccines** –They are derived from attenuated or vaccine strains of viruses. Using replication-competent vectors often results in a more robust immune response than with replication-incompetent vectors, since they replicate within the vaccinated individual and trigger an innate immune response. Among COVID-19 vaccine candidates, replication-competent vectors have been engineered to express the spike protein in measles vaccine strain vectors, influenza virus-based vectors, vesicular stomatitis virus (VSV), and Newcastle disease virus (NDV) (World Health Organization 2020, Zhu et al., 2020 and Case et al., 2020). NDV-based vectors propagate to high titers in eggs and could be produced using the global influenza vaccine production pipeline; they could also be given intranasally to stimulate mucosal immunity at the site of viral entry.

● **DNA vaccines** – These vaccinesconsist of plasmid DNA that contains mammalian expression promotors and the target gene, so that the target protein is expressed in the vaccine recipient. Large quantities of stable plasmid DNA can be generated in *Escherichia coli*, which is a major production advantage. However, DNA vaccines are often of low immunogenicity and need special delivery devices, such as electroporators, which limit their use. Further, DNA vaccines must reach the nucleus to be transcribed to messenger RNA (mRNA) so proteins can be generated to stimulate an immune response.

● **RNA vaccines** – RNA vaccines were the first vaccines for SARS-CoV-2 to be produced and represent an entirely new vaccine approach. Once administered, the RNA is translated into the target protein, which is intended to elicit an immune response. The mRNA remains in the cell cytoplasm and does not enter into the nucleus; mRNA vaccines do not interact with or integrate into the recipient's DNA. These vaccines are produced completely *in vitro,* which facilitates production. However, some of the vaccines must be maintained at very low temperatures, complicating storage. The anti- SARS-CoV-2 vaccine that are currently approved by WHO are listed in Table 2 below:

Table 2 List of vaccines approved by World Health Organization based on data up to December 1, 2021.

Platform	Vaccine Name	Developer/Manufacture	Countries approved in
RNA	mRNA-1273	Moderna	78 Countries
RNA	BNT162b2	Pfizer/BioNTech	112 Countries
Non-replicating viral vector	Ad26.COV2.S	Janssen (Johnson & Johnson)	85 Countries
Non-replicating viral vector	AZD1222	Oxford/AstraZeneca	127 Countries
Non-replicating viral vector	Serum Institute of India	Oxford/AstraZeneca	47 Countries
Inactivated	Covaxin	Bharat Biotech	12 Countries
Inactivated	BBIBP-CorV (Vero Cells)	Sinopharm (Beijing)	72 Countries
Inactivated	CoronaVac	Sinovac	46 Countries

CHAPTER III
MATERIALS AND METHODS

3.1 Nasopharyngeal swab collection from patients

Nasopharyngeal (NP) and oropharyngeal (OP) specimens were collected from individuals using synthetic swabs and were placed in 3 ml lysis buffer solution provided by the kit manufacturer (Diagnovital®, RTA Laboratories Inc.) (Figure 10). Viral lysis buffer is a viral transport medium (VTM) which inactivates the sample to be noninfectious and provides stabilization of SARS-CoV-2 RNA for up to 48 hours. Combined NP and OP specimens were collected with a single swab in a single tube in order to maximize test sensitivity. Collected swab samples were transported to the DESAM COVID-19 PCR Laboratory and stored at 4°C until use.

A **B**

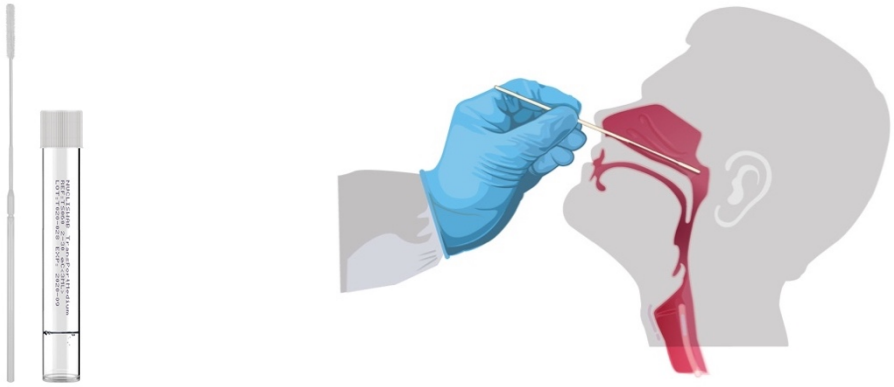

Figure 10. (A) Swab and VTM for COVID-19 testing (B) Collection of a NP clinical specimen performed by a trained healthcare provider

3.2 Processing of nasal swabs for nucleic acid extraction

Collected samples in VTM were vortexed for 20 seconds for lysis and the lysates were stored at -20°C until further analysis (Figure 11). Vortexing of samples was performed in a biosafety level 2 (BSL2) cabinet to avoid droplet contamination. RT-qPCR was performed directly from the lysate samples.

Figure 11. Preparation of NP samples for RT-PCR by vortexing

3.3 RT-PCR for the detection of SARS-CoV-2

Diagnovital® (RTA Laboratories Inc, SARS-CoV-2 Real-Time PCR Kit v2.0 Istanbul-Turkey), a commercial kit was used for patient screening. Diagnovital® is designed to detect SARS-CoV-2-specific N1 and N2 nucleocapsid genes. A total of 15 µl mastermix containing the primer-probe mix and 5 µl sample was used for each reaction (Table 3). An internal control (RNAseP gene) amplification was monitored in the RT-qPCR to assess the quality of sample collection and RT-qPCR analysis. A no template (negative) control and positive controls, both provided with the kit, were included in each RT-qPCR run. Samples that had a cycle threshold (Ct) value of less than 40 were considered positive.

Table 3. Components of Diagnovital RT-PCR kit for SARS-CoV-2 detection

Component	**Volume (µl)**
PCR Master Mix	15
RNA Isolate/PC/NTC	5
Total	**20**

All reactions were performed using Insta Q96TM Plus Real-time PCR Detection System (HiMedia Laboratories Pvt. Ltd.). The amplification protocol was followed as the manufacturer's instructions and is given in Table 4. Data collection was performed in FAM and HEX channels.

Table 4.

Step	Cycles	Temperature	Duration
Reverse Transcription	1	45°C	10 minutes
Initial Denaturation	1	95°C	2 minutes
Amplification	45	95°C	10 seconds
		55°C*	30 seconds

3.4 Interpretation of RT-PCR results

For a sample to be considered positive for SARS-CoV-2, its amplification in the FAM channel (N1/N2 genes) and a Ct value was required.

For a sample to be considered negative for SARS-CoV-2, the FAM channel (N1/N2 genes) was required not to give a Ct value or amplification. The internal control in the HEX channel (RNAseP) was required to give a Ct value (<40 cycles) to ensure that the sample material of suitable quality was present. For the positive control, a positive Ct in the FAM channel should be observed. The Ct value for the positive control should be 28<Ct<32. The interpretation of the RT-PCR results is summarized in Table 5.

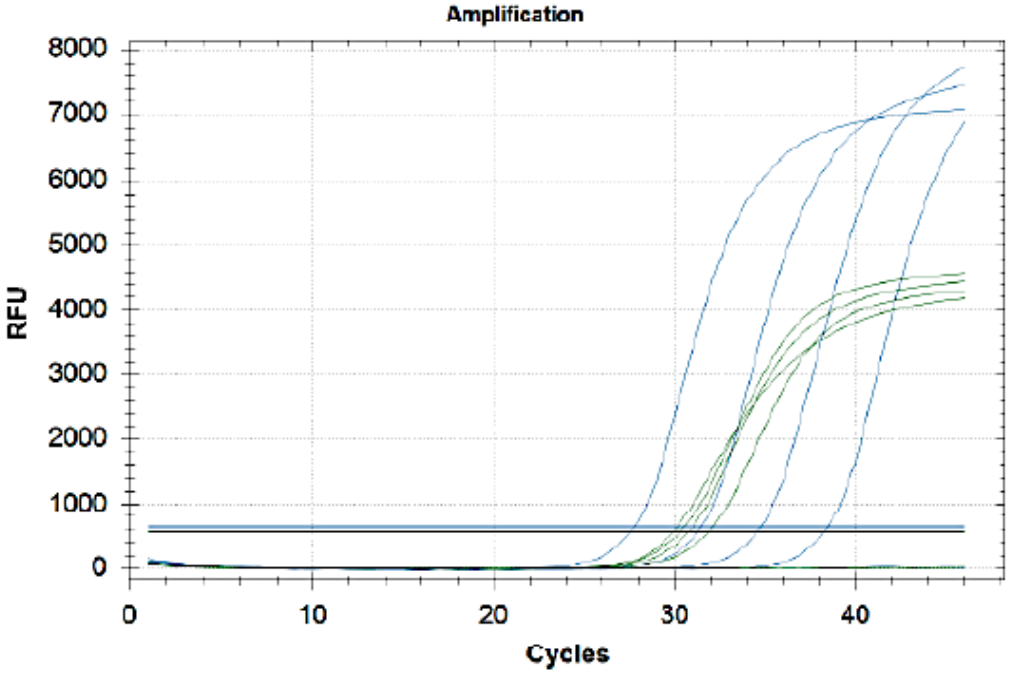

Figure 12. Blue curves: positive samples in the FAM channel, green curves: internal control in the HEX channel, curves running straight: negative control

Table 5.

N1-N2 (FAM)	RNAseP (HEX)	Interpretation
+	-	The sample is considered positive for SARS-CoV-2.
+	+	The sample is considered positive for SARS-CoV-2.
-	+	Only the target sequence for the internal control was amplified. The sample is considered negative for SARS-CoV-2.
-	-	PCR was inhibited, results are invalid.
+	-	Expected result for the positive control
-	-	Expected result for the negative control

3.5 Collection of COVID-19 patient data

Ct values obtained from RT-PCR results of tests performed at DESAM COVID-19 PCR Laboratory at Near East University between January and October 2021 were collected for this study. Ethical approval was taken from the Institutional Review Board (YDU/2021/97-1438) at Near East University.

A total of 3436 positive RT-PCR result was included in this study. Samples included both patients admitted to Near East University Hospital and individuals screened through routine governmental screening implemented by the Turkish Republic of Northern Cyprus Ministry of Health. Ct value per patient was recorded and categorized according to month. A comparison of Ct values for each positive RT-PCR result was compared monthly in order to evaluate any statistically significant trends across months. Particularly, January 2021 in which the Alpha B.1.1.7 variant was first detected in Northern Cyprus until June-July 2021 in which all months in between were dominated by the Alpha B.1.1.7 variant was compared in terms of Ct values and hence viral load of patients.

3.6 Statistical analysis

Statistical analysis of data was performed using the SPSS Statistics Version 23.0 (IBM, Armonk, New York). One-way ANOVA was used to compare the Ct means of ten consecutive months in order to determine whether there is statistical evidence that the population means were significantly different. A Bonferroni post-hoc test was applied. A p value of <0.05 was considered statistically significant.

CHAPTER IV
RESULTS

4.1 Sample characteristics

This thesis was conducted at the DESAM COVID-19 PCR Testing Laboratory at Near East University Hospital from January to October 2021. A total of 3436 SARS-CoV-2 positive patients were included in this study. These included those from patients admitted to Near East University Hospital and samples obtained from patients screened through routine governmental screening. Out of the total samples, 39.30% were obtained from patients admitted to Near East University Hospital, while 60.70% of the samples were collected from patients detected through routine governmental screening (Figure 13).

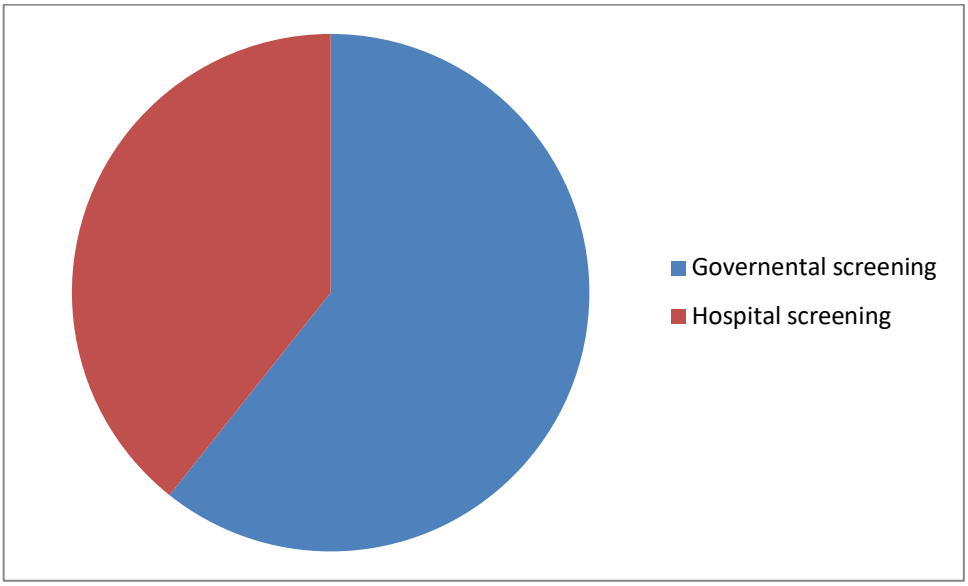

Figure 13. Percentage of total samples obtained from Near East University Hospital and from routine governmental COVID-19 PCR screening

4.2 Distribution of SARS-CoV-2 positive samples

The frequency of the number of COVID-19 positive cases from January to October 2021 was evaluated and recorded. The number of cases varied widely across these months and peak positivity was recorded in August 2021 (Figure 14).

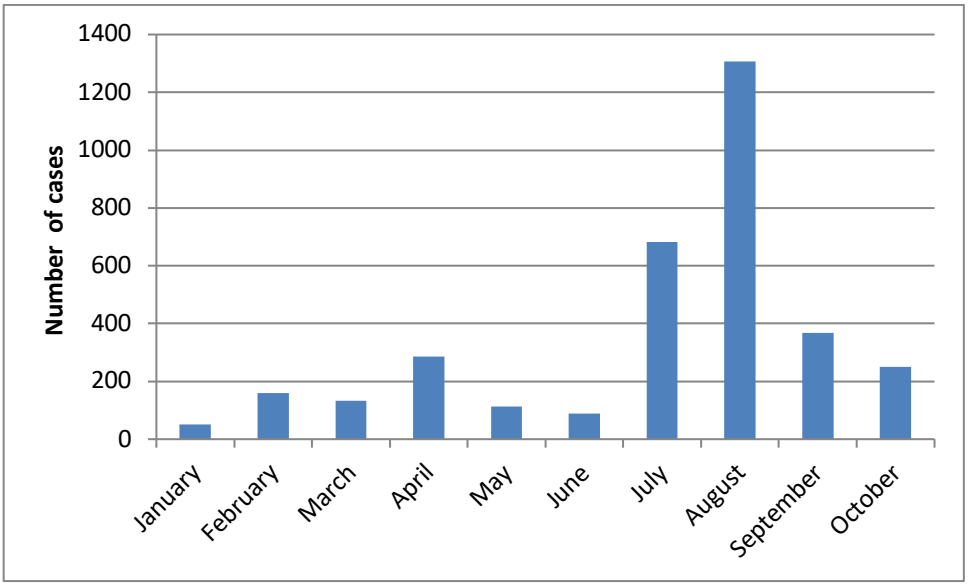

Figure 14: Monthly COVID-19 cases from January to October 2021

4.3: Evaluation of SARS-CoV-2 RT-PCR Ct values across months

Ct values of SARS-CoV-2 RT-PCR results per patient per month was collected and analyzed. The minimum and maximum Ct values per month as well as the monthly mean Ct values is given in Table 6. The lowest Ct value was detected as 9.53 in August 21 and the highest Ct value was observed in March 21 as 39.01.

Table 6. Minimum and maximum SARS-CoV-2 RT-PCR Ct values per month

Month	Number of samples (n)	Minimum Ct value	Maximum Ct value	Mean Ct value	Standard deviation
Jan 21	50	11.70	36.52	25.0396	4.65293
Feb 21	160	13.72	39.01	26.9836	5.88228
March 21	133	14.31	39.89	27.8951	6.18638
April 21	285	15.31	38.83	26.3655	4.36029
May 21	113	14.82	35.80	26.6231	4.99166
June 21	89	14.80	35.57	23.5767	4.78160
July 21	682	11.83	35.37	21.7826	4.78082
August 21	1306	9.53	32.69	22.9066	3.59565
September 21	368	11.46	29.65	21.8216	3.59043
October 21	250	16.23	33.73	24.6435	3.14801

4.4. Categorization of monthly SARS-CoV-2 RT-PCR Ct values and viral load as high, moderate and low

The obtained Ct values were categorized as Ct<25 (high viral load), Ct 25-30 (moderate viral load) or Ct>30 (low viral load). As apparent in Table 7, the monthly Ct values showed a decreasing trend towards June, July, August and September 2021 with most of the Ct values being below 25. Similarly, a higher proportion of cases with Ct values above 30 were observed in February, March, April 2021 compared to later months. Overall, an increase in cases with low Ct value and high viral load from January was detected followed by a rapid increase in July and August.

Table 7: Total number of positive COVID-19 cases cross tabulation based on Ct range

CT RANGE ACROSS MONTHS				
	<25	**25-30**	**>30**	**TOTAL**
January	27	15	8	50
February	58	50	52	160
March	45	32	56	133
April	117	113	55	285
May	39	44	30	113
June	58	20	11	89
July	500	139	43	682
August	955	317	34	1306
September	293	75	0	368
October	133	103	14	250
TOTAL	2225	908	303	3436

4.5 Statistical analysis of monthly Ct values

The mean of values per month (mean of viral load per month) was analyzed to determine whether or not there was statistical evidence of significant difference between the population means. Table 8 indicates that significant differences were observed. Compared to January 2021, in which majority of detected SARS-CoV-2 was WT, statistically lower Ct values were observed in March, July, August and September 2021. The first case of SARS-CoV-2 Alpha B.1.1.7 variant was declared in January 2021 in Northern Cyprus, which became the dominant variant through July 2021. The first SARS-CoV-2 Delta B.1.617.2 variant was announced in July 2021in Northern Cyprus and was the dominant variant in positive COVID-19 cases in August, September and October 2021.

Table 8: Mean differences in monthly Ct values and statistical analysis

	Mean Difference to January	Standard Error	Significance	95% Confidence interval	
				Lower Bound	Upper Bond
January	February	.68618	0.209	4.1834	.2954
	March	.70257	0.002*	5.1484	.5627
	April	.64937	1.000	3.4451	.7934
	May	.71936	1.000	3.9311	.7641
	June	.74852	1.000	.9799	3.9057
	July	.62052	0.000*	1.2319	5.2821
	August	.61031	0.022*	.1413	4.1248
	September	.63834	0.000*	1.1347	5.3012
	October	.65612	1.000	1.7452	2.5374

* statistically significant

The median of Ct values across the months were also evaluated and recorded. The positive COVID-19 cases with the lowest Ct value (≤ 25 Ct values) and highest viral load was recorded in the month of July (Figure 15) in which SARS-CoV-2 Alpha B.1.1.7 variant was dominant. It is important to note that this coincides with the emergence of SARS-CoV-2 Delta B.1.617.2 variant.

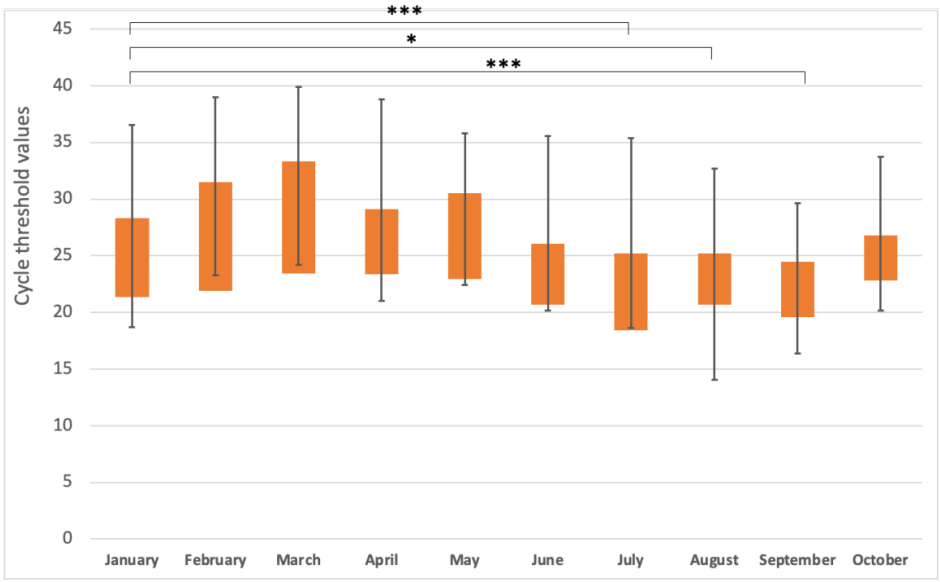

Figure 15: Monthly Ct values of COVID-19 positive cases from January to October 2021 (*p<0.05, **p<0.01, *p<0.001)

CHAPTER V
DISCUSSION

Since its emergence in 2019, COVID-19 has spread worldwide leading to global pandemic. Currently, the pandemic surge largely depends on the observation of local cases and mortality rates, this has a tendency of lagging changes in transmission rates by several weeks or concealed by changes in testing capacity (Hay et al., 2020). Due to the high availability of RT-PCR data and critical nature of the COVID-19 pandemic, there is a rising interest in the need to explore the possibility that population distribution of Ct values can be used as potential indicators to determine the dynamics of local outbreak and potential surge. The detection of viral nucleic acid using RT-PCR is considered a gold standard for the diagnosis of COVID-19. One of the advantages of using RT-PCR for the screening of individuals with SARS-CoV-2 is minimization of hands-on time and the accuracy of the results over other conventional methods. For accurate infection control, estimating the number of infected persons and determining the dynamics of spread in the population is critical. The Ct values as a proxy of viral load and a possible indicator for infectivity and severity of infection has been investigated by several studies worldwide. The Ct value is the number of polymerase chain reaction cycles at which the fluorescence signal of a particular sample exceeds background level. Ct values are inversely proportional to the amount of viral nucleic acids present in a sample. A lower Ct value indicates a higher amount of target nucleic acid in a sample.

In this study, Ct values of COVID-19 positive tests between January and October 2021 have been analyzed. Study results shows a significantly higher level of positive cases in August 2021 compared to other months. The lowest Ct value was also recorded in the same month. A recent cohort study reported that samples with lower Ct values had higher infectivity (Jaafer et al., 2021).

Evaluation of Ct values of SARS-CoV-2 RT-PCR results per patient per month showed that minimum Ct value per month was detected in August 2021, while the maximum Ct value per month was observed in March 2021. A recent study suggested that a decrease in median cycle threshold value of a population may be an indication of an

increased level of transmission (Hay et al., 2021). Furthermore, the cross tabulation of total number of positives cases in this study indicated that 64.75% (n=2225) of all positive cases (n=3436) had a Ct value of <25 with a peak in the month of July and August. This statistically lower Ct values and peak positivity of cases in of August can be associated with certain epidemiological factors such as increased human interaction between the populations; which could be as a result of increased social activities in the months of the summer holiday. Considering that between February and July 2021 SARS-CoV-2 Alpha B.1.1.7 was dominant, the statistically higher viral load can be attributed to the Alpha B.1.1.7 variant. Compared with December 2020 (data not shown) and January 2021 in which WT SARS-CoV-2 was dominant, Alpha B.1.1.7 variant was found to exhibit lower Ct values and hence a higher viral load in June and July compared to WT virus. Interestingly, SARS-CoV-2 Delta B.1.617.2 variant was first reported in Northern Cyprus in July 2021 and was dominant through August, September and October. This B.1.617.2 Delta variant was named a variant of concern by the WHO and has been reported to be more infectious (Allen et al., 2021). The statistically lower Ct values of COVID-19 tests in September 2021 compared to January 2021 can be attributed to the B.1.617.2 Delta variant. Several recent studies have reported differences in viral load between variants. Teyssou et al. have shown that the Delta variant presented a significantly higher viral load (median 7.69 [6.58–8.94]) than the historical variants (median 7.02 [5.26–8.15]) and the Beta variant (median 7.26 [6.10–8.37]) (Teyssou et al., 2021). Another study has also demonstrated that the RT-PCR tests positive for Alpha and Beta/Gamma variants exhibited significantly Ct values compared to WT SARS-CoV-2 (Roquebert et al., 2021). It is important to note that in cases of mixed infection by two different viruses, SARS-CoV-2 Ct values can be higher with a lower viral load compared to cases with a single infection with SAR-CoV-2. This can be attributed to competition for nutritional requirements to support proliferation for the viruses in infected niche (human cells).

A significantly high proportion of positive cases and low Ct values can be used as an early indicator of a surge. The median of Ct values across the months indicated that the lowest Ct value and highest viral load was recorded in the July. The median Ct value has also been suggested as a useful indicator for predicting a pandemic surge (Zein et al., 2020). In addition, a recent study by Aranha et al., reported that individuals with a high Ct

value or lower viral load could clear SARS-CoV-2 viral RNA in short period. This study further observed that 66.4% of the cases with a Ct value greater than or equal to 31 could clear the viral load within 14 days of initial detection, while a mere 20.8% of the individuals with a Ct value of less than or equal to 25 were able to clear the viral load within the same time. This observation can be an indicator of the fact that determining Ct values and viral load can be of utmost importance and aid the understanding of COVID-19 prognosis.

Some of the strengths of this study include the fact that all the RT-PCR analyses were conducted at a single laboratory using standardized testing protocols and test kits, and that a large number of positive COVID-19 tests were acquired for this study. Furthermore, the samples used for this study were not limited to single medical center, but included samples collected from an entire geographical area. Conversely, a weakness of this study is the lack of access to data that accounts for the effects of certain host factors such as: age, gender, vaccination status, immunocompromised individuals, underlying disease such as asthma on Ct values of infected individuals.

The RT-PCR testing data features examined by this study will contribute to and affect the use of population-based Ct values in predicting disease dynamics and infectivity timeline in a geographical location.

CHAPTER VI
CONCLUSION AND RECCOMENDATION

An ideal diagnosis of SARS-CoV-2 infection is dependent on several critical factors. These factors include; appropriate selection and availability of tools and techniques. For a better control over the COVID-19 pandemic, a global collaborative effort is required. This study has utilized RT-PCR technique for the analysis of Ct value which is considered an indicator for viral load in the population. Ct value also highlights the effect of the emergence of certain SARS-CoV-2 variants on viral load and by extension, infectivity and possible transmissibility of the virus based on the high proportion of positive samples during specific months. This can be used as an indicator of a possible surge and can also be of great importance for understanding infectivity timeline and viral load dynamics of infected individuals.

The current study concluded that the emergence of the Alpha variant has caused significantly lower Ct values, hence higher viral load in infected individuals in Northern Cyprus. In order to monitor the effects of Delta and Omicron variants on the viral load in the population, further data collection within the recent months and analysis are required.

With the continuous emergence of SAR-CoV-2 virus variants, it is critical to keep track of the virus and its behavioral patterns to promote and enable time preventative measures.

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APPENDICES

Appendix A

Turnitin Similarity Report

thesis jan 2022

ORIGINALITY REPORT

15%	%	15%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Mohammad Alkhatib, Valentina Svicher, Romina Salpini, Francesca Alessandra Ambrosio et al. "SARS-CoV-2 Variants and Their Relevant Mutational Profiles: Update Summer 2021", Microbiology Spectrum, 2021 Publication	2%
2	Mei-Yue Wang, Rong Zhao, Li-Juan Gao, Xue-Fei Gao, De-Ping Wang, Ji-Min Cao. "SARS-CoV-2: Structure, Biology, and Structure-Based Therapeutics Development", Frontiers in Cellular and Infection Microbiology, 2020 Publication	2%
3	Edward C. Holmes, Stephen A. Goldstein, Angela L. Rasmussen, David L. Robertson et al. "The origins of SARS-CoV-2: A critical review", Cell, 2021 Publication	2%
4	Gulten Tuncel, Mahmut Cerkez Ergoren, Buket Baddal, Pinar Tulay et al. "Comparison of RT-qPCR results of different gene targets for SARS-CoV-2 in asymptomatic individuals	2%

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2018	SECOND CLASS UPPER DIVISION	GODFREY OKOYE UNIVERSITY, ENUGU, NIGERIA	MEDICAL MICROBIOLOGY

3. ACADEMIC EXPERIENCE

PERIOD	TITLE	DEPARTMENT	UNIVERSITY
APRIL-SEPTEMBER 2017	RESEARCH-INDUSTRIAL TRAINING	GENETICS AND GENOMIC BIOTECHNOLOGY	NATIONAL INSTITUE OF BIOTECHNOLOGY DEVELOPMENT AGENCY, ABUJA, NIGERIA,
NOVEMBER 2018-OCTOBER 2019	RESEARCH OFFICE ASSISTANT	EDUCATION SUPPORT SERVICES	FEDERAL MINISTRY OF EDUCATION, ABUJA, NIGERIA.

4. FIELD OF INTERESTS

FIELDS OF INTERESTS	KEY WORDS
MEDICAL RESEARCH, MOLECULAR VIROLOGY	RESEARCH, VIROLOGY

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi :25.11.2021
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Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Doç. Dr. Buket Baddal'ın sorumlu araştırmacısı olduğu, YDU/2021/97-1438 proje numaralı ve **"Temporal dynamics of viral load in respiratory tract specimens of COVID-19 patients in Northern Cyprus"** başlıklı proje önerisi kurulumuzca değerlendirilmiş olup, etik olarak uygun bulunmuştur.

Prof. Dr. Şanda Çalı
Yakın Doğu Üniversitesi
Bilimsel Araştırmalar Etik Kurulu Başkanı

Kurul Üyesi	**Toplantıya Katılım**	**Karar**
	Katıldı(✓)/ Katılmadı(X)	*Onay(✓)/ Ret(X)*
Prof. Dr. Tamer Yılmaz	✓	✓
Prof. Dr. Şahan Saygı	✓	✓
Prof. Dr. Nurhan Bayraktar	✓	✓
Prof. Dr. Mehmet Özmenoğlu	✓	✓
Prof. Dr. İlker Etikan	✓	✓
Doç. Dr. Mehtap Tınazlı	✓	✓
Doç. Dr. Nilüfer Galip Çelik	✓	✓
Doç. Dr. Emil Mammadov	✓	✓
Doç. Dr. Ali Cenk Özay	✓	✓