EVALUATION OF GENSCRIPT CPASS AND IMMUSAFE COVID+ ASSAYS FOR THE DETECTION OF NEUTRALIZING ANTIBODY TITERS AGAINST SARS-CoV-2 IN INDIVIDUALS WITH SECOND AND BOOSTER DOSES OF BNT162b2

FARAAZLIN BINTI SALLEH

UNIVERSITI SAINS MALAYSIA

2023

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by

FARAAZLIN BINTI SALLEH

Dissertation submitted in partial fulfillment of
the requirements of the degree of
Master of Science (Biomedicine) Mixed Mode

AUGUST 2023

ACKNOWLEDGEMENT

First and foremost, I am deeply grateful to Allah Almighty, for whom His blessing gives me health, abilities, and the courage to complete this research. The research project will not be able to be completed without any support or assistance. I am truly grateful for all the hard work and dedication I have received from many parties.

I would like to express my sincere gratitude to my supervisor, Assoc. Prof. Dr. Rapeah Suppian, for her continuous support and guidance during this semester of completing my research project. Besides, I would like to thank my co-supervisor, Dr Aini Syahida Mat Yassim, for enlightening my research journey during the research proposal. The great experience and knowledge poured out by my supervisor and co-supervisor have always encouraged me to complete my research, even in the face of adversity. I would also like to thank my research project coordinator, Dr Wong Weng Kin, for providing me with invaluable resources and guidance I needed throughout the writing of my thesis.

Finally, I would like to express my gratitude to my parents and friends for their unwavering support and belief throughout every stage of my journey. A big thanks to both of my parents for sending me back and forth to university until the end of my journey as a master's degree student. I want to express my gratitude to my research partner, Wani Husna Binti Mohd Izani, for her assistance and encouragement throughout my laboratory sessions and research journey.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

~	About
%	Percentage
>	More than
^{0}C	Degree Celsius
µg	Microgram
µL	Microliter
ACE2	Angiotensin-converting enzyme 2
BMI	Body mass index
BSL-3	Biosafety laboratory level 3
C1q	Complement component 1q
C3d	Complement component C3d
COVID-19	Coronavirus disease 2019
cVNT	Conventional virus neutralization test
D2-2	Second dose after two weeks
D3-2	Third dose after two weeks
DMV	Double membrane vesicle
ELISA	Enzyme linked immunosorbent assay
et al.	*et alii* – 'and others'
ER	Endoplasmic reticulum
ER-GIC	Endoplasmic reticulum – Golgi intermediate compartment
FDA	Food and drug administration
HREC	Human Research Ethics Committee
HRP-RBD	Horseradish peroxidase-receptor binding domain

kb	Kilobase
KKM	Kementerian Kesihatan Malaysia
Ig	Immunoglobin
IMR	Institutes of Medical Research
mL	Millilitre
MOH	Ministry of Health
mRNA	Messenger ribonucleic acid
NA	Not applicable
NIH	National Institutes of Health
nM	Nanomolar
nsps	Non-structural protein
NTD	N-terminal domain
OD	Optical density
ORF1a	Open reading frame 1a
ORF1b	Open reading frame 1b
pp1a	Protein phosphatase 1a
pp1b	Protein phosphatase 1b
RBD	Receptor binding domain
rpm	Rotations per minute
PBS	Phosphate buffered saline
PRNT	Plaque reduction neutralization test
SAB	Streptavidin-biotin
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SD	Standard deviation
TBS	Tris-buffered saline

TEMRA	Terminally differentiated effector memory cells
TMB	3,3’,5,5’-Tetramethylbenzidine
TMPRS22	Transmembrane serine protease 2
USM	Universiti Sains Malaysia
WHO	World Health Organization

LIST OF APPENDICES

PENILAIAN UJIAN GENSCRIPT CPASS DAN INMUSAFE COVID+ DALAM PENGESANAN MENEUTRALKAN TITER ANTIBODI TERHADAP SARS-CoV-2 DALAM INDIVIDU DOS KEDUA DAN PENGGALAK BNT162b2

ABSTRAK

Wabak penyakit Coronavirus telah menyebabkan berjuta-juta kematian, dan orang ramai juga kehilangan pekerjaan di seluruh dunia. Vaksinasi meningkatkan peneutralan titer antibodi dan mengurangkan penyebaran SARS-CoV-2. Antibodi yang meneutralkan menghalang interaksi virus dengan menyekat pengikatan antara domain pengikat reseptor SARS-CoV-2 dan permukaan reseptor perumah. Matlamat utama kajian ini adalah untuk menilai ujian GenScript cPass dan ImmuSAFE COVID+ untuk mengesan titer antibodi yang meneutralkan menggunakan sampel yang dikumpul daripada dos kedua dan penggalak vaksin BNT162b2. Ujian GenScript cPass dan ImmuSAFE COVID+ ialah ujian peneutralan semasa di pasaran. Penilaian ujian peneutralan menunjukkan peningkatan ketara secara statistik dalam antibodi peneutralan selepas dos penggalak menggunakan GenScript cPass. Sebaliknya, ImmuSAFE COVID+ menunjukkan penurunan dalam meneutralkan antibodi selepas dos penggalak. Data demografi antara umur dan jantina terhadap peneutralan titer antibodi juga dinilai. Tiada korelasi antara jantina dan umur ke arah meneutralkan antibodi. Kajian itu menyimpulkan bahawa GenScript cPass dan ujian ImmuSAFE COVID+ menunjukkan keupayaan dalam menentukan peneutralan titer antibodi selepas vaksinasi.

EVALUATION OF GENSCRIPT CPASS AND IMMUSAFE COVID+ ASSAYS FOR THE DETECTION OF NEUTRALIZING ANTIBODY TITERS AGAINST SARS-CoV-2 IN INDIVIDUALS WITH SECOND AND BOOSTER DOSES OF BNT162b2

ABSTRACT

The coronavirus disease outbreak has caused millions of deaths and widespread unemployment globally. Vaccination boosts neutralizing antibody titers and reduce the spread of SARS-CoV-2 virus. Neutralizing antibodies inhibit the interaction of the virus by blocking the binding between the receptor binding domain of SARS-CoV-2 and the host receptor surface. The primary aim of this study was to evaluate GenScript cPass and ImmuSAFE COVID+ assays for detecting neutralizing antibody titers using samples collected from second and booster doses of the BNT162b2 vaccine. GenScript cPass and ImmuSAFE COVID+ assays are the current neutralization tests on the market. The evaluation of neutralization tests demonstrated a statistically significant increase in neutralizing antibodies after the booster dose using GenScript cPass. In contrast, ImmuSAFE COVID+ showed a decline in neutralizing antibodies after the booster dose. The demographic data between age and gender against neutralizing antibody titers was also evaluated, showing no correlation between gender and age towards neutralizing antibodies. The study concludes that GenScript cPass and ImmuSAFE COVID+ assays demonstrate the ability in determining neutralizing antibody titers post-vaccination.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The world was caught off guard at the end of December 2019 by a dreadful outbreak that claimed the lives of more than six million individuals by July 2023. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus that causes the disease known as coronavirus disease 2019 (COVID-19). The virus is known as 'corona' due to the crown-like spikes on the virus receptor. The rapid emergence of the virus has led to changes in the population due to the need for home quarantine and stricter surveillance. As a consequence of the continuous issue with COVID-19, millions of people have been forced out of their jobs, and numerous people have also lost their lives across the world.

COVID-19 was first identified in Wuhan, Hubei, China, in November 2019 and was initially reported to the World Health Organization (WHO) in December 2019. The Wuhan-Hu strain of SARS-CoV-2 is the original strain of COVID-19, also known as the wild type. The original strain has been mutated into different variants known as Alpha, Beta, Delta, and Omicron. These variants present different characteristics based on transmissibility, likelihood, and disease severity. As mentioned by Yaugel-Novoa *et al.* (2022), COVID-19 has a diverse spectrum of clinical manifestations, encompassing cases ranging from asymptomatic or mild illness to more severe cases necessitating hospitalization with oxygen treatment and in some instances, resulting in fatality.

The global emergence of COVID-19 has led to the discovery of different types of vaccines to fight against SARS-CoV-2. Vaccines from Pfizer-BioNTech, Moderna, Johnson & Johnson, AstraZeneca, Sputnik V, Sinovac, and Novavax are among the common vaccinations available on the market. In Malaysia, Pfizer-BioNTech, Sinovac, AstraZeneca, and CanSino are the most recommended vaccines by the Ministry of Health (MOH). Nevertheless, Pfizer-BioNTech and Sinovac are the most popular vaccines in Malaysia as these vaccines are widely available and more accessible due to the subsidized cost by the government. The availability of vaccines at no cost is a significant incentive that motivates the majority of the population to be vaccinated.

A booster vaccination programme was initiated in Malaysia in October 2021 involving homologous (Pfizer-BioNTech) and heterologous (Sinovac) vaccines. The primary purpose of the booster vaccination is to boost antibody titers, which in turn increases protection and reduces the severity of the infection from COVID-19. The necessity of booster doses can be reinforced by a previous study by Naaber *et al.* (2021), which stated that after two doses of the Pfizer-BioNTech vaccine, immune responses were weak, and the frequency of non-responders was lower among older people. The need for a booster dose after the second dose is recommended, as Zheng *et al.* (2023) mentioned that vaccination provides temporary protection against diseases, with the efficacy of vaccines beginning to decrease within six to eight months. Aside from that, antibody titers were significantly lower three to six months after the second dose, with a low CD4+ T cell response and higher levels of senescent CD8+ TEMRA cells (Naaber *et al.*, 2021).

In addition, evaluation of neutralization antibodies is essential to determine whether the immune response from vaccination or natural infection exhibits elevated antibody titers. Antibodies capable of neutralizing the SARS-CoV-2 virus serve as a marker that interferes with its transmission. Most neutralization tests are used for analytical and clinical use to identify individual needs to get the booster dose if the level of neutralizing antibody titers is low. Various neutralization tests are available on the current market for the dual use of tracking COVID-19 immunity and the development of vaccines. The serological assays can be selected based on the highest percentage of accuracy and sensitivity. Many studies compared the performance of different neutralization tests, such as the DiaSOrin SARS-CoV-2 S antibody ELISA kit and the Quidel Quickvue SARS-CoV-2 antibody test. The plaque reduction neutralization test (PRNT) is well-known as the gold standard for neutralizing antibody detection. However, PRNT is highly sensitive and must be conducted in a high biosafety laboratory level 3 (BSL-3) precaution. Therefore, neutralizing antibody test kits with ELISA-based methods is increasingly preferred in modern society and extensively implemented in clinical settings, surpassing the conventional gold standard method as a potential future alternative (Li *et al.*, 2021).

The current virus neutralization antibody detection tests available in the market are GenScript cPass and ImmuSAFE COVID+ neutralization antibody detection kits. The National Institutes of Health (NIH) developed the GenScript cPass assay, whereas Sengenics was the company that developed the Krex technology by utilizing the ImmuSAFE COVID+ assay. These technologies are commonly used to identify misfolded proteins and evaluate the effectiveness of the various vaccines against the immune response to COVID-19. The ImmuSAFE COVID+ test has 100% sensitivity

and specificity and has been validated by the Institutes of Medical Research (IMR). The sensitivity and specificity of ImmuSAFE technology make the test an ideal platform for validating the efficacy of various vaccines (BERNAMA, 2021). Comparatively, Jung *et al.* (2021) reported that GenScript cPass had a sensitivity of 96% and a specificity of 100% for the detection of neutralizing antibodies.

1.2 Problem statement

The problem that this study addresses is the availability of the current virus neutralization tests. There are different types of neutralization antibody detection kits available in the market. In a technologically advanced world, neutralizing antibody detection kits characterized by enhanced efficiency, automation, high-throughput, and expedited processes are highly preferred in clinical settings. GenScript cPass and ImmuSAFE COVID+ assays are the current tests available to detect neutralizing antibodies. However, the evaluation between the efficiency of these two virus neutralization tests is yet to be resolved. More research needs to be published on the detection of neutralizing antibodies utilizing ImmuSAFE COVID+. Moreover, most existing studies on GenScript cPass primarily focus on its comparison with conventional virus neutralization test. More published journals are needed to address the use of the ImmuSAFE COVID+ assay to determine the presence of neutralizing antibodies. Therefore, the effectiveness of the detection of neutralizing antibodies in GenScript cPass and ImmuSAFE COVID+ assays was evaluated using samples of second and booster doses of the BNT162b2 vaccine. This study will prove the efficiency of neutralization tests in detecting neutralizing antibodies, corroborating previous studies showing a significant difference after booster dose.

1.3 Rationale of the study

The study of SARS-CoV-2 remains pertinent despite the change of COVID-19 from a pandemic to an endemic state. The mutation of SARS-CoV-2 can reduce the effectiveness of vaccines. Due to the mutations of SARS-CoV-2, understanding different types of variants is essential to observe in terms of the transmissibility and severity of the virus for tracking COVID-19. Although the Wuhan-Hu variant is no longer dominant, studying the evolution of the original wild-type Wuhan-Hu strain is still essential.

Furthermore, the comparison of neutralization antibody titers between second and booster doses provides significant evidence for vaccination strategies based on GenScript cPass and ImmuSAFE COVID+ assays. The detection of neutralizing antibodies is important for predicting the effectiveness of the vaccine after a booster dose. According to Atmar *et al.* (2022), the effectiveness of messenger RNA (mRNA) vaccines is related to the binding of neutralizing antibody titers. The neutralizing antibody titers were expected to be higher in booster dose despite different serological COVID-19 assays. Besides, the comparison between different types of neutralization assays allows researchers to evaluate the assays in terms of cost-effectiveness, speed, and safety of different neutralization assays. The monitoring of vaccination response is essential, especially for older people. Therefore, the research aids in advocating for booster vaccinations and educating public health by providing strong evidence of the high neutralizing antibodies after booster shots. Other than that, the study of age and gender influences on the neutralizing antibody titers can provide vaccination strategies of different age groups and genders for further studies.

1.4 Objective of the study

The general objective of the study was to evaluate GenScript cPass and ImmuSAFE COVID+ assays for the detection of neutralizing antibody titers against SARS-CoV-2 in individuals with second and booster doses of the BNT162b2.

The specific objectives include:

1. To determine neutralizing antibody titers using GenScript cPass and ImmuSAFE COVID+ assays in second and booster doses of serum samples.
2. To analyse neutralizing antibody titers changes in correlation with age and gender of second and booster doses.

1.5 Overview of the study

The methodology of the study is as shown below.

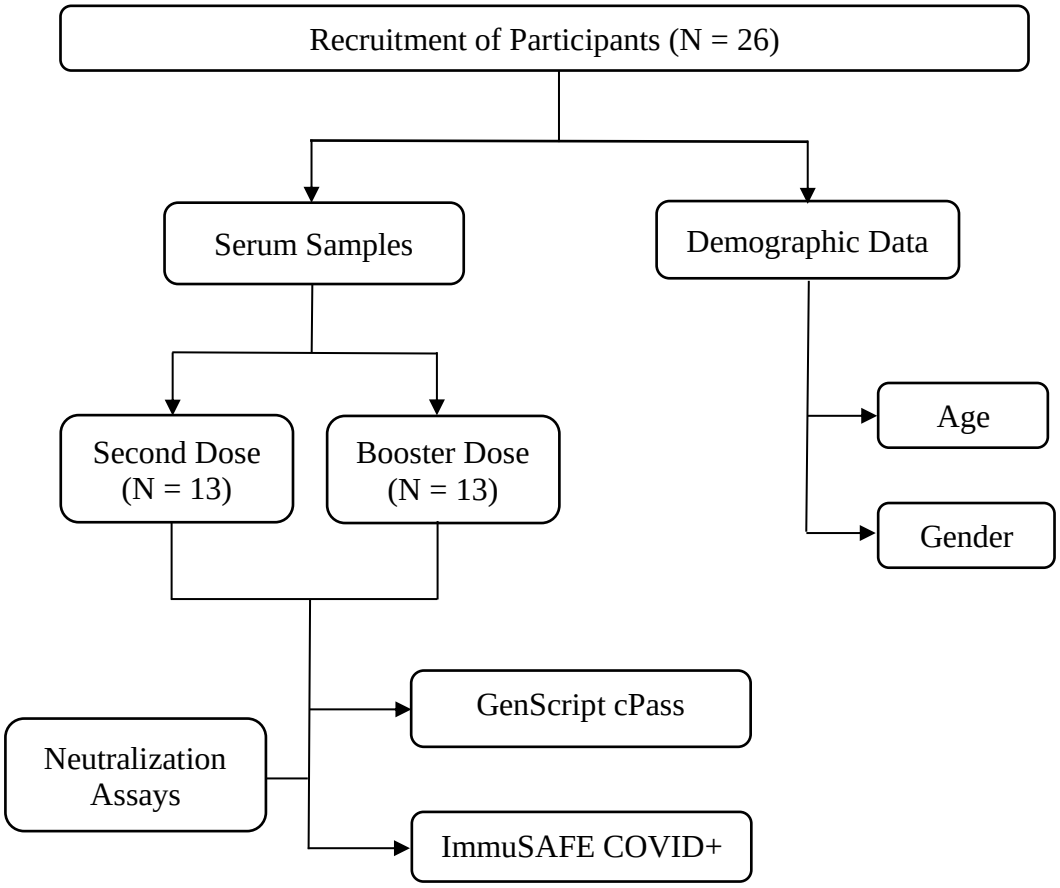

Figure 1.1 Study flowchart

CHAPTER 2

LITERATURE REVIEW

2.1 SARS-CoV-2

2.1.1 Structure of SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) contains a single-stranded positive-sense RNA, also known as a molecular messenger responsible for protein synthesis. The genome of SARS-CoV-2 is larger (27 –32 kb) than any other RNA virus (Wang *et al*., 2020). The structure of SARS-CoV-2 can be divided into four major protein parts, which are spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. The receptor binding domain (RBD) spike protein is fundamental for virus-host cell interactions.

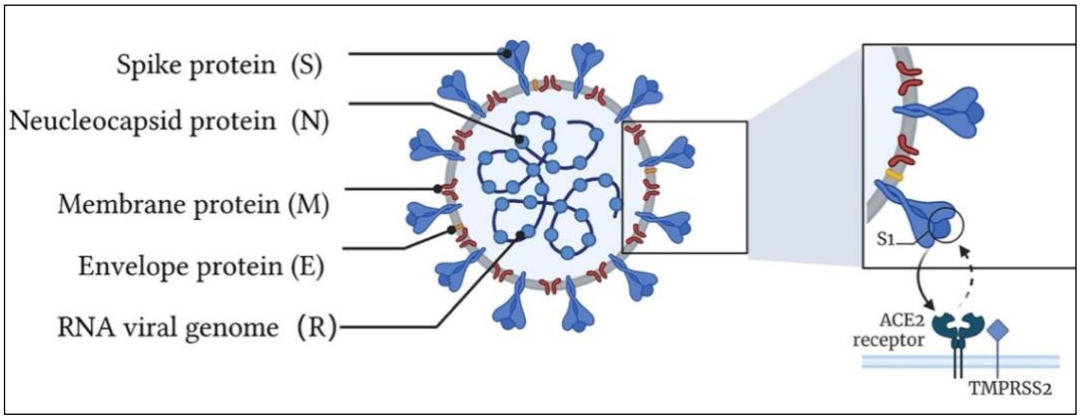

Figure 2.1 Structure of SARS-CoV-2
(Zhu *et al*., 2022)

As Magazine *et al.* (2022) mentioned, the spike protein is responsible for target recognition, cellular entrance, and endosomal escape. The spike protein is divided into the S1 subunit and the S2 subunit, where the S1 subunit contains a C-terminal RBD and an N-terminal domain (NTD). The S1 subunit recognizes and binds to the target

cell, while the S2 subunit mediates the viral cell membrane fusion. Based on Figure 2.1, the S1/S2 protease cleavage site is the part of the SARS-CoV-2 virus that is between the S1 and S2 subunits. There are two serine proteases responsible for the entry of the virus, which are furin protease and type II transmembrane serine protease (TMPRS22) located on the surface of the human host receptor, such as nasal, bronchial, and gastrointestinal epithelium. The furin protease is essential for cleaving the S1 subunit of the spike protein, while TMPRS22 is responsible for cleaving the S2 subunit of the spike protein. The binding between RBD and angiotensin-converting enzyme 2 (ACE2) activates TMPRSS2, allowing the virus to enter the cell. In other words, TMPRSS2 works by cleaving the S1/S2 protease and making it easier for the S1 subunit to bind to the ACE receptor and the S2 subunit to help the virus fuse with the host cell membrane (de Freitas Santoro *et al*., 2021).

In addition, spike protein is glycosylated and essential for the primary target in the mutation of the SARS-CoV-2 virus. These mutations have a significant impact because RBD will bind to the ACE2 during viral infections. Based on previous studies, the mutations on the RBD resulted in a more significant interaction between RBD and ACE2 receptor (Wong *et al.,* 2023). Compared to the Wuhan Hu variant, the Omicron variant contains more than 30 mutations. The effect of mutations causes the virus to become easier to bind to human cells in the Omicron variant compared to the Wuhan Hu variant due to the high number of mutations in the spike protein. For example, the D614G is a mutation in the spike protein discovered in all primary circulating strains of SARS-CoV-2 and is associated with a significant increase in the infectivity of the virus (Magazine *et al.,* (2022). The mutations cause the virus to respond differently based on its transmissibility, severity of diseases, and infection rates. Thus, the

Omicron variant is more transmissible than the original SARS-CoV-2 Wuhan Hu variant.

2.1.2 Transmission of SARS-CoV-2

The transmissibility of the SARS-CoV-2 virus varies depending on natural infection and vaccination. ACE2 is the primary receptor for the viral spike protein during COVID-19 infection and is directly associated with virus fusion and entry into the host cell (Chen *et al.,* 2023). The most common way for SARS-CoV-2 to enter the body is through the respiratory tract, where droplets from an infected person coughing or sneezing enter the body.

The virus can infect the host cell membrane by adhering RBD to ACE2 through two pathways: cell surface entry or endosomal entry. Entry through the cell surface refers to early infection, a more common pathway for the SARS-CoV-2 virus. Endosomal entry refers to late infection, a less common and slower pathway than the cell surface entry.

Figure 2.2 shows the transmission of the virus through the cell surface entry pathway. Firstly, the SARS-CoV-2 enters the body and binds to the ACE2 of the host receptor. Then, furin protease cleaves the outer part of the spike protein, namely the S1 subunit. The cleavage of the S1 subunit exposes the inner core of the spike protein, the S2 subunit. TMPRSS2 cleaves the S2 subunit allowing the spike protein to unfold and anchor into the host cell membrane.

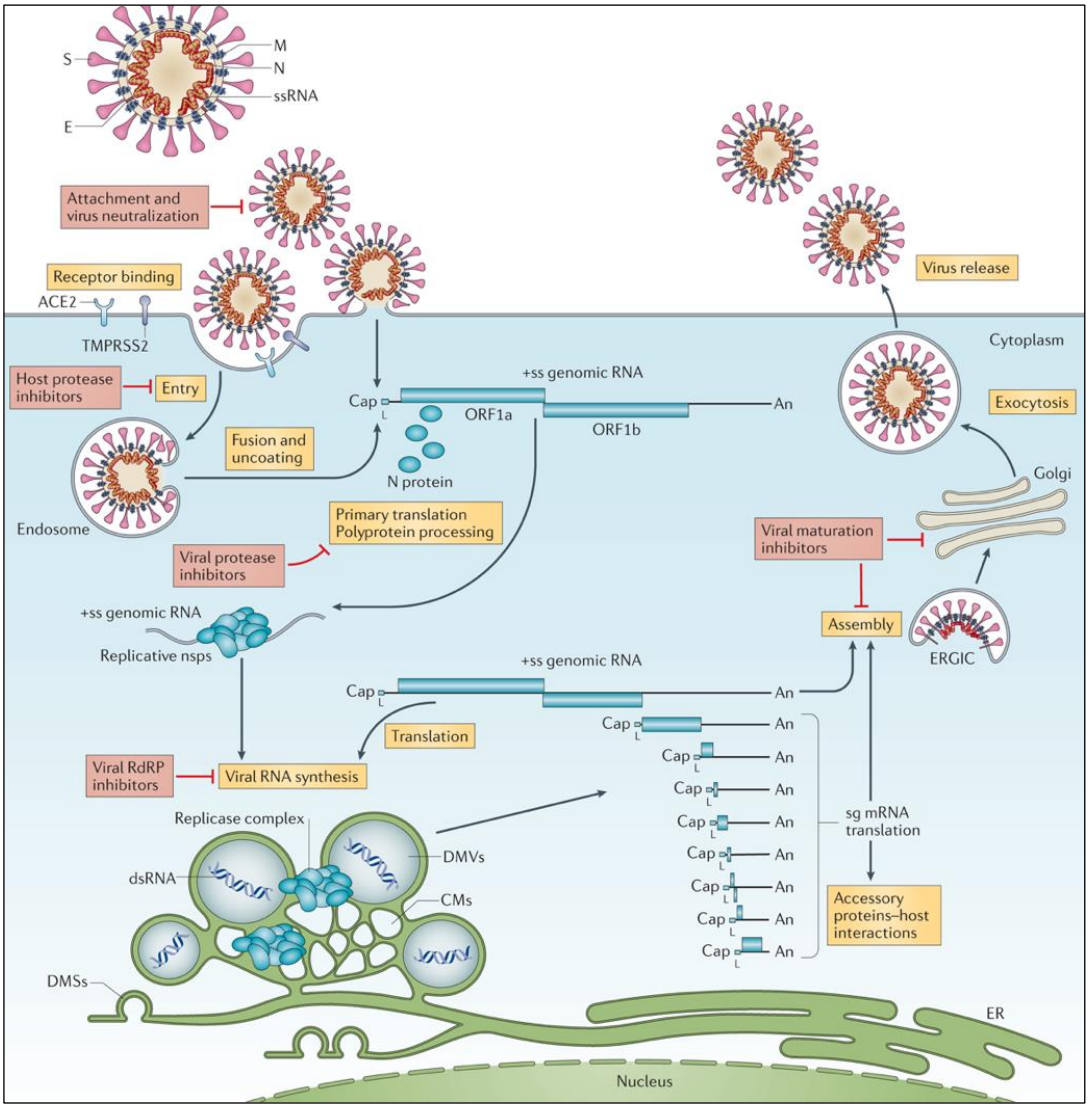

Figure 2.2 Transmission of SARS-CoV-2 via cell surface entry (Pizzato *et al.*, 2022)

As Wang *et al.* (2020) mentioned, the proteases in the host cell cut the S2 subunit cleavage site of the spike glycoprotein, releasing the proteins needed to fuse the virus and host cell through irreversible conformational changes. The viral genome encounters ribosomes in the cell and starts translating its genetic code. The ORF1a and ORF1b genes are translated, resulting in a long protein chain containing polyproteins pp1a and pp1b. The polyproteins co-translationally and post-translationally into the individual non-structural proteins (nsps) that comprise the viral replication and

transcription complex (V'kovski *et al.,* 2020). The nsps are embedded into the membrane and create a double membrane vesicle (DMV) structure. The DMV is a safe and enclosed environment suitable for viral genomic RNA replication. Then, translated structural undergo translocation into the endoplasmic reticulum (ER) membranes and travel via the ER to reach the Golgi intermediate compartment (ER-GIC). The interaction with nucleocapsids generates genomic RNA causing budding into secretory vesicular compartments. Therefore, virions are released from the infected cell by exocytosis.

2.2 Humoral immune response to SARS-CoV-2

The synthesis of IgA, IgG, and IgM antibodies refers to the response to the SARS-CoV-2 virus. B cells activate the humoral immune response, which triggers the humoral immune response to produce antigen-specific antibodies to fight against the virus. The memory B cells are essential in developing long-term and widespread humoral memory responses. Besides, memory B cells evolve in response to repeated antigen exposure, which reactivates to produce antibodies in circulation (Kotaki *et al.,* 2023). According to Yaugel-Novoa *et al.* (2022), the fragment antigen-binding region and the crystallizable fragment are the mechanisms that neutralize antibodies that block the entry of the virus into the host cell).

Figure 2.3 shows a humoral immune response to SARS-CoV-2 regarding age, gender, disease severity, and previous immunity. The humoral immune response can be divided into mucosal and systemic. The production of antibody titers is produced differently based on the disease severity and individual characteristics such as age and gender. In the systemic humoral immune response, Figure 2.3 concluded that higher

levels of ACE2 were found in men than women. Besides, the levels of specific IgG, IgA, and IgM in the serum of older individuals are high, but the levels of mucosal IgA are low in these individuals (Yaugel-Novoa *et al.*, 2022). This contrasts the situation in young individuals with lower anti-SARS-CoV-2 antibody titers in their serum but more significant levels of mucosal IgA.

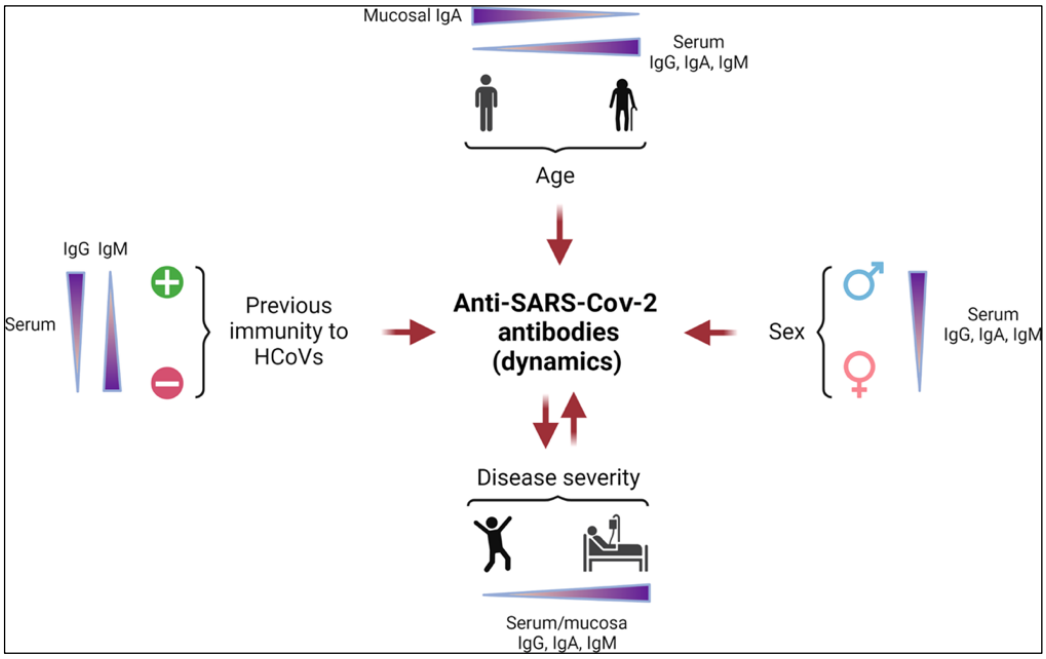

Figure 2.3 Humoral immune response to SARS-CoV-2 (Yaugel-Novoa *et al.*, 2022)

However, the systemic humoral immune response shows high antibody titers in older people with immunosenescent and comorbid conditions. In the systemic humoral immune response in Figure 2.4, IgA is the predominant isotype. At the same time, IgM levels in the blood decline significantly after one month, whereas particular IgA and IgG levels remain stable for more than six weeks. The IgG is a vital antibody that can be identified for up to a year. However, asymptomatic individuals have lower SARS-

CoV-2 antibodies and lose their specific IgG antibodies faster than symptomatic individuals.

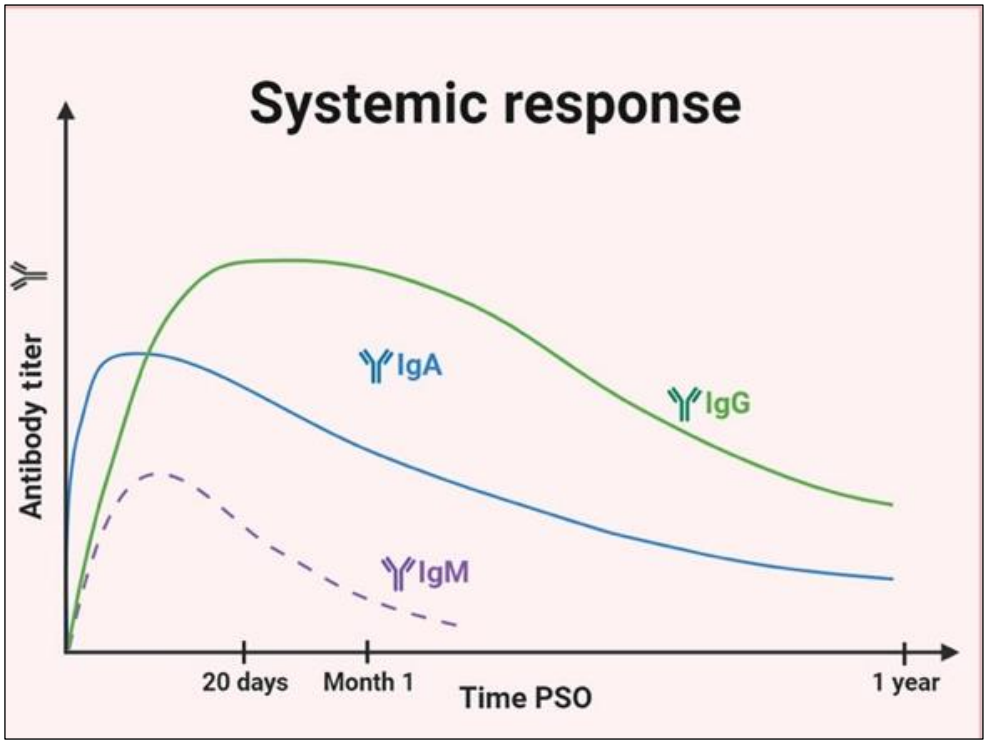

Figure 2.4 Antibody titers in systemic response (Yaugel-Novoa *et al.*, 2022)

2.3 Humoral immune response to COVID-19 vaccination

Understanding the humoral immune response is important to develop more effective vaccines to combat novel variations. Most COVID-19 vaccines stimulate humoral immunity, identifying the spike protein as the target to prevent the virus from binding with the hACE2. According to Wong *et al*. (2023), the mutations in the spike protein of the receptor binding domain (RBD) pose a concern as they have the potential to impede the efficacy of current vaccination regimens.

Individuals who had previously been infected and received the second dose of BNT162b2 developed higher titers of anti-spike IgG and IgA antibody titers compared to never-infected individuals. C1q and C3d are proteins in the complement cascade against the COVID-19 infection. As specified by Yaugel-Novoa *et al*. (2022), plasma from people who had been vaccinated showed high amounts of C1q and C3d binding to spike-specific antibodies or RBD-specific antibodies, which was different from convalescent plasma. The vaccination causes higher amounts of IgG1 and IgG3 than natural infection does, and by the fact that these isotypes are more effective at activating the complement cascade. Other than that, it has been shown that previously infected individuals have enhanced neutralizing antibodies after five months, in contrast to individuals who have never been infected and have just had a booster vaccination one month prior (Yaugel-Novoa *et al.*, 2022). Therefore, vaccinations generate high anti-SARS-CoV-2 IgG concentrations in previously infected individuals. Vaccination produces more sialylated, fucosylated, and galactosylated antibodies, notably IgG1, which induce less severe disease than natural infection (Yaugel-Novoa *et al.*, 2022). Vaccination does not entirely prevent the spread of SARS-CoV-2 infection, but it plays an important role in reducing the spread of SARS-CoV-2.

2.4 Neutralizing antibodies

2.4.1 Neutralizing and binding antibodies

Neutralizing antibodies are produced in response to natural infection and vaccine administration. During SARS-CoV-2 infection, neutralizing and non-neutralizing antibodies are produced (Chen *et al.*, 2023). It is imperative in fighting against SARS-CoV-2 regardless of whether individuals have received the Moderna, Pfizer/BioNTech, CanSino or Sinovac vaccines. Roeder *et al.* (2022) mentioned that vaccinated individuals with the wild-type SARS-CoV-2 spike protein-encoding mRNA are more likely to undergo breakthrough infections due to decreased neutralizing antibodies. However, breakthrough infections are more likely to boost the neutralizing antibodies in vaccinated individuals.

According to L'Huillier *et al.* (2021), individuals infected with SARS-CoV-2 will produce neutralizing antibodies within three weeks of infection. Never-infected individuals are prone to have weaker neutralizing antibodies compared to previously infected individuals. The RBD of the SARS-CoV-2 correlates with the neutralizing antibody titers. In comparison between never-infected and previously infected individuals, there was a moderate correlation between full spike-binding IgG antibodies and neutralizing antibodies and a strong correlation between the levels of the spike RBD and N-terminal domain IgG antibodies and neutralizing antibodies (Narowski *et al.*, 2022). Although there is a robust correlation between the RBD and neutralizing antibodies, the neutralizing antibodies tend to lose their efficacy as several variants of concern arise. The neutralizing antibodies function as weapons by binding to the viral antigen of SARS-CoV-2 and effectively blocking the interaction between the virus and host cells, thereby impeding the progression of the viral infection. According to Chen *et al.* (2023), the binding of neutralizing antibodies with a virus

triggers steric effects by preventing conformational changes. Besides, neutralizing antibodies is prominent as a tool for bolstering vaccine development and facilitating convalescent plasma treatment (Jung *et al.*, 2021). Neutralizing antibodies that exhibit high titers and persistence play a crucial role in establishing herd immunity (Chen *et al.*, 2023). The higher the neutralizing antibody, the higher the protection rate. The other important factor of neutralizing antibodies can be seen in Figure 2.5, showing the binding of antibodies in an infected individual.

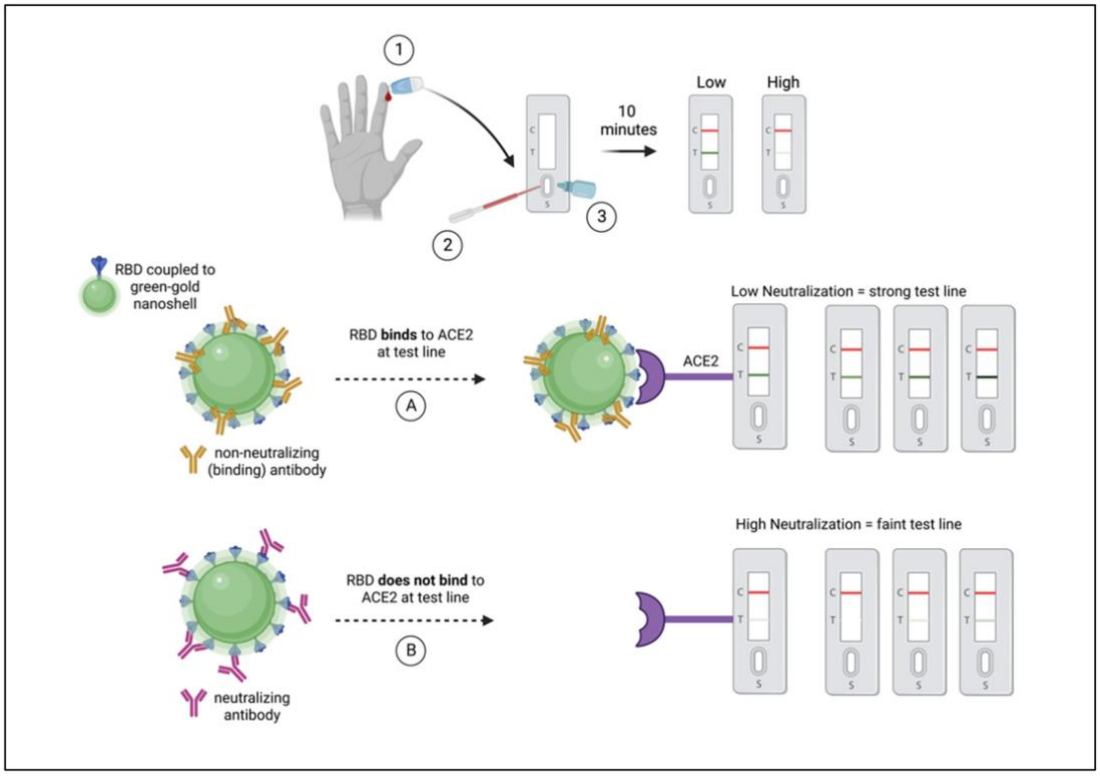

Figure 2.5 Binding antibody and neutralizing antibody (Roeder *et al.*, 2022)

The binding antibody (non-neutralizing) binds between the SARS-CoV-2 virus and the ACE2. However, the binding antibodies cannot neutralize the virus by blocking the interaction between the RBD-ACE2. Therefore, the serological assay shows a strong test line, resulting in low neutralization antibodies. On the other hand, the

neutralization antibodies that bind between the RBD and ACE2 can block the entry of the virus into the host receptor. The blocking of the RBD-ACE2 shows a faint test line in the serological test, showing that high neutralization antibodies are found in the serum. The comparison in Figure 2.5 shows the significant presence of neutralizing antibodies through natural infection or vaccination. However, neutralizing antibody titers react differently according to different types of vaccines. For example, both mRNA-1273 and BNT162b2 give a high efficacy against SARS-CoV-2, but mRNA-1273 vaccination presented higher neutralizing antibody titers than BNT162b2 vaccination due to the factors of dose contents, vaccine formulations, and intervals between doses (Shurrab *et al.*, 2022).

2.5 Virus neutralization test

2.5.1 Plaque reduction neutralization test

Plaque reduction neutralization test (PRNT) is the gold standard for detecting and quantifying neutralizing antibodies. It is also referred to as a conventional virus neutralization test, essential in validating sample diagnostic tests and establishing correlations among the community. As mentioned by Müller *et al.* (2021), the endpoint titers of PRNT can be calculated for every serum sample at the chosen percentage reduction in viral activity, commonly 50% or 90%.

In PRNT, serum samples are mixed with infectious virus particles and subsequently cultured on a cell monolayer, allowing for the observation of cytopathic effects (Hofmann *et al.*, 2021). The accuracy of PRNT is higher than other surrogate virus neutralization tests. The PRNT does not require immediate counting and store chemically fixed cells for extended periods, allowing for multiple calculations of plaque numbers and enhancing the authenticity and accuracy of the test (Chen *et al.*, 2023). Nevertheless, PRNT is time-consuming, requires a BSL-3 due to the use of live viruses and is not suitable for performing large-scale testing as it requires a skilled workforce.

2.5.2 GenScript cPass surrogate virus neutralization test

GenScript cPass is the first surrogate virus neutralization test (sVNT) approved by the FDA. The high-tech in GenScript cPass replicates the interaction between the virus and the host cell, resulting in a roughly 1.5-hour detection of total neutralizing antibodies compared to PRNT, which takes several days. It detects samples containing neutralizing antibodies capable of selectively inhibiting the interaction and viral entry between the virus into the host cell. The GenScript cPass does not require live viruses or extensive training and can be conducted without BSL-3 precautions. Table 2.1 shows previous publications studied using GenScript cPass. The principle of GenScript cPass refers to the HRP-conjugated RBD which is bound explicitly to the ACE2 receptor, as shown in Figure 2.6. The ELISA plate of GenScript cPass contains the ACE2 receptor, while the neutralizing antibody from the serum, and HRP-conjugated RBD is the reagent provided by GenScript cPass.

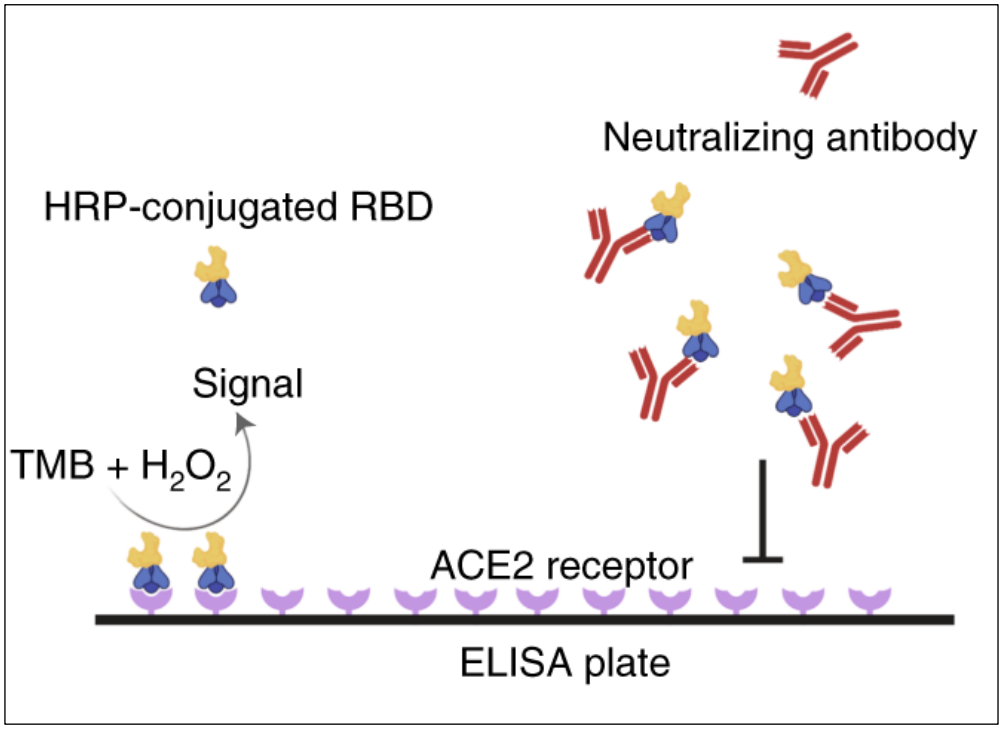

Figure 2.6 Principal procedure of GenScript cPass (Chen *et al.*, 2023)

Table 2.1 Previous publication using GenScript cPass

Summary	Publication Year	Author
Measured neutralizing titers in patients who recovered from COVID-19 or have been vaccinated	2021	Jung *et al.*
Observed neutralising antibody titres as antibody levels wane and viral variants emerge.	2022	Muecksch *et al.*
Determined the correlations between the levels of anti-RBD IgG and neutralizing antibodies (NAbs) against SARS-CoV-2 variants in vaccinated subjects	2022	Takheaw *et al.*
Assessed whether a commercially available neutralisation assay (cPass, Genscript) provides a genuine measure of SARS-CoV-2 neutralisation	2021	Murray *et al.*
Evaluated the performance of the first commercial surrogate virus neutralization test (GenScript cPass) against SARS-CoV-2 PRNT in convalescent and vaccinated individuals	2022	Hofmann *et al.*
Developed a platform to quantify NAb levels faster, at lower cost, and with better efficiency.	2022	Chiu *et al.*
Provided data on anti-S1 spike total IgG and neutralizing antibodies in vaccinated and non-vaccinated, including naturally infected	2022	Batchi-Bouyou *et al.*

2.5.3 ImmuSAFE COVID+ protein array

ImmuSAFE COVID+ is a multi-antigen, multi-domain, fully quantitative and qualitative test that uses Sengenics' patented Krex technology. It detects the quantity and location of the antibody and reports the presence of neutralizing antibodies. Besides, ImmuSAFE COVID+ provides a more accurate determination of seroprevalence by targeting six different domains of SARS-CoV-2. According to Shaffaf & Ghafar-Zadeh (2021), ImmuSAFE COVID+ has the ability to evaluate the vaccination response in patients by distinguishing between antibodies produced in reaction to the vaccine and those generated from a prior illness. It has been developed to minimize cross-reactivity by selectively targeting certain domains of the SARS-CoV-2 virus, enhancing the accuracy of determining seroprevalence. The full-length spike antigen featured by ImmuSAFE COVID+ explains the 100% sensitivity and specificity of the neutralization assay, as the immune system can recognize the full-length spike antigen than the RBD antigen.

CHAPTER 3

METHODOLOGY

3.1 Ethical approval

Samples and data acquisition were approved by the Human Research Ethics Committee (HREC) of the Universiti Sains Malaysia (USM), Kelantan as attached in Appendix 1. Participants signed informed consent before being recruited into the study.

3.2 Study participants

The samples were collected by venipuncture of 26 participants at USM, collecting eight mL of blood into serum separator tubes. The samples from those with only two doses of the BNT162b2 vaccination were collected two weeks later (D2-2). On the other hand, samples from individuals who had a booster dose of BNT162b2 were collected after the third dosage, two weeks post-vaccination (D3-2). The data was obtained by observing individuals who had either been infected within the past six months or had never been infected. Serum preparation was performed by aliquoting into microcentrifuge tubes and stored at -80 °C until ready to run the neutralization assay.

3.3 Sample size of participants

The study population (n = 26) consists of two groups: individuals who received only two doses of the BNT162b2 vaccine and individuals who received a booster dose of the BNT162b2 vaccine. The sample size calculation was performed using G*Power,

as attached in the appendices. However, a formal sample size calculation was not conducted for this research due to the small sample size. Among the total samples, 13 were selected from individuals who had never been infected and had only received two doses of the BNT162b2 vaccination, while 13 samples were selected from individuals who had never been infected and had received a booster dose of the BNT162b2 vaccination. The age range of the chosen samples from both groups varied from 25 to 55 years old. However, for a longitudinal analysis, only three identical individuals from each group had serum taken from the second and booster doses of the BNT162b2 vaccination.

In this study, samples were chosen only from never-infected individuals. The neutralizing antibody titers are lower in never-infected individuals compared to individuals who had been infected because neutralizing antibody titers in never-infected individuals are exposed only to vaccination and not to the natural infection. Thus, samples from never-infected individuals were used to compare the differences in neutralizing antibody titers before and after the booster dose of BNT162b2 vaccination.