EVALUATION OF GENSCRIPT CPASS AND IMMUSAFE COVID+ ASSAYS IN DETECTION OF SARS-CoV-2 NEUTRALISING ANTIBODY TITRES IN CORONAVAC SECOND AND BNT162b2 BOOSTER DOSES

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

COVID-19	Coronavirus disease 2019
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
WHO	World Health Organisation
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
mRNA	Messenger RNA
et al.	and others
PRNT	Plaque reduction neutralisation test
sVNT	Surrogate virus neutralisation test
FDA_EUA	Food and Drug Administration Emergency Use Authorization
HRP	Horseradish peroxidase
RBD	Receptor-binding domain
RBD-HRP	HRP-conjugated RBD
ACE2	Angiotensin-converting enzyme 2
ExoN	Exoribonuclease
BSL-2	Biosafety level 2
BSL-3	Biosafety level 3
ELISA	Enzyme-linked immunosorbent assay
hACE2	Human ACE2 protein
pVNT	Pseudo-virus neutralisation test
MARii	Malaysia Automotive, Robotics and IoT Institute
IMR	Institute of Medical Research
Ig	Immunoglobulin

HREC	Human Research Ethics Committee
USM	Universiti Sains Malaysia
%	Percentage
µL	Microliter
mL	Millilitre
nm	Nanometre
nM	Nanomolar
°C	Degree Celsius
NA	Not applicable
TMB	3,3’,5,5’-Tetramethylbenzidine
SAB	Serum Albumin Buffer
D2-2	Second dose of CoronaVac after two weeks
D3-2	Third dose (booster) dose of BNT162b2 after two weeks
OD	Optical density
NAb	Neutralising antibodies
ID_{50}	Serum dilution needed to lower 50% of the ACE2 binding signal
VOC	Variants of concern
LFIA	Lateral flow immunoassay

LIST OF APPENDICES

PENILAIAN UJIAN GENSCRIPT CPASS DAN IMMUSAFE COVID+ DALAM PENGESANAN TITER ANTIBODI PENEUTRALAN TERHADAP VIRUS SARS-CoV-2 DALAM DOS KEDUA CORONAVAC DAN PENGGALAK DOS BNT162b2

ABSTRAK

Wabak penyakit koronavirus (COVID-19) pada tahun 2020 telah disebabkan oleh koronavirus sindrom pernafasan akut teruk 2 (SARS-CoV-2). Pandemik ini telah menjejaskan ramai orang di seluruh dunia menyebabkan vaksin COVID-19 dihasilkan sebagai usaha untuk menghentikan pandemik. Vaksin yang diluluskan oleh kerajaan Malaysia termasuk Sinovac CoronaVac dan Pfizer-BioNTech BNT162b2. Vaksinasi boleh meningkatkan tahap antibodi peneutralan dan memberikan perlindungan terhadap jangkitan COVID-19. Antibodi peneutralan adalah penting dalam memantau keberkesanan vaksin terhadap virus SARS-CoV-2. Beberapa ujian telah dikeluarkan untuk mengesan titer antibodi peneutralan dalam kalangan individu. Tujuan kajian ini adalah untuk menilai dua ujian komersial iaitu GenScript cPass dan ImmuSAFE COVID+ dalam mengesan titer antibodi peneutralan SARS-CoV-2. Kedua-dua ujian menggunakan 26 jumlah sampel serum dos kedua CoronaVac (n = 13) dan dos penggalak BNT162b2 (n = 13). Kajian ini juga menentukan titer antibodi peneutralan antara perempuan dan lelaki di kalangan belia dalam dos kedua dan penggalak. Hasil kajian menunjukkan titer antibodi peneutralan lebih tinggi dalam sampel serum dos penggalak BNT162b2 berbanding dos kedua CoronaVac. Analisis data demografi menunjukkan titer antibodi peneutralan dalam wanita adalah lebih tinggi daripada lelaki di kalangan belia dalam sampel dos kedua dan penggalak. Kajian ini

merumuskan bahawa kedua-dua ujian boleh dipercayai dalam mengesan antibodi peneutralan dalam sampel serum selepas vaksinasi.

EVALUATION OF GENSCRIPT CPASS AND IMMUSAFE COVID+ ASSAYS IN DETECTION OF SARS-CoV-2 NEUTRALISING ANTIBODY TITRES IN CORONAVAC SECOND AND BNT162b2 BOOSTER DOSES

ABSTRACT

Coronavirus disease 2019 (COVID-19) outbreak in 2020 was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The pandemic has affected a lot of people worldwide, resulting in COVID-19 vaccines being developed as an effort to stop the pandemic. The vaccines approved by the Malaysian government include Sinovac CoronaVac and Pfizer-BioNTech BNT162b2. Vaccination can increase the level of neutralising antibodies and protect against COVID-19 infections. Neutralising antibodies are essential in monitoring the effectiveness of the vaccines against SARS-CoV-2. Several assays have been developed to detect neutralising antibody titres in individuals. The purpose of this study was to evaluate the two commercialized assays, GenScript cPass and ImmuSAFE COVID+ tests in detecting SARS-CoV-2 neutralising antibody titres. Both assays used a total of 26 serum samples of CoronaVac second (n = 13) and BNT162b2 booster (n = 13) doses. The study also determined neutralising antibody titres between males and females among young adults in second and booster doses. The results showed the titre of neutralising antibodies was higher in BNT162b2 booster dose than in CoronaVac second dose serum samples. The analysis of the demographic data showed neutralising antibody titres in females were higher than in males among young adults in second and booster dose samples. The study concluded that both assays were reliable in detecting neutralising antibodies in serum samples following vaccination.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

In 2020, the entire world, including Malaysia, faced the coronavirus disease 2019 (COVID-19) outbreak that was caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The virus was discovered in Wuhan, a city in China, in 2019. The World Health Organisation (WHO) (2020) swiftly declared the infection as a pandemic on 11 March 2020. This declaration was announced considering COVID-19 was contagious and infected a lot of people throughout the world (Farid *et al*., 2022). According to WHO (2023), more than 7.68 billion confirmed cases and more than 6.9 million fatalities had been reported globally as of June 2023. In Malaysia, COVID-19 has resulted in more than 37,164 deaths and a total of 5,120,581 confirmed cases as of 12 July 2023 (Ministry of Health Malaysia, 2023).

Direct contact with an intermediary host, such as wild pangolins, can result in the direct transmission of SARS-CoV-2 to humans, and the virus can quickly spread from one person to another (Elengoe, 2020). Consuming meat infected with SARS-Cov-2 may also contribute to virus transmission. COVID-19 is a zoonotic disease where an animal virus mutates, infects and replicates within the human body. Infected people can spread the virus through respiratory droplets. Consequently, sneezing and coughing render the virus airborne, putting non-infected people at risk of being infected with the infection (Sharma *et al*., 2021). SARS-CoV-2 is a single-stranded RNA virus that can cause symptoms ranging from mild to severe. Huang *et al*. (2020) reported the common symptoms are fevers, coughs and tiredness. Some infected people may

experience a variety of symptoms, including headaches, muscle pains, sore throat, rhinorrhoea (runny nose) or diarrhoea. This infection may also cause severe pneumonia, organ damage, acute respiratory tract infection, and septic shock, resulting in the patient's death (Huang *et al*., 2020).

A significant effort was made to develop COVID-19 vaccines that could be used all over the world to stop the pandemic as a result of the rapid spread of SARS-CoV-2 infection. There have been numerous vaccines designated to prevent the disease, control the transmission of diseases, and hinder future occurrences. In Malaysia, eight vaccines against COVID-19 are currently approved for use (Covid Vaccine Tracker, 2022). The vaccines are available in three types: inactivated or weakened virus, RNA and DNA, and viral vector vaccines. Examples for each vaccine are inactivated virus (Sinovac CoronaVac, Sinopharm/Beijing BBIBP-CorV, and Bharat Biotech BBV152), mRNA (Pfizer/BioNTech BNT162b2 and Moderna mRNA-1273), and non-replicating viral vector (Janssen/Johnson & Johnson Ad26.COV2.S, Oxford-AstraZeneca AZD1222, and CanSino Ad5-nCoV) vaccines (Covid Vaccine Tracker, 2022). The vaccines have also been administered in SARS-CoV-2 immunisation programmes globally since 2020. The Sinovac CoronaVac vaccine contains an inactivated or weakened strain of the virus. Despite not being a disease-causing agent, it still triggers an immune reaction. The Ministry of Health Malaysia (2022) reported CoronaVac as the second most widely used vaccine brand, with a percentage of 29.8. Inactivated vaccines have been extensively used to immunise individuals in numerous developing nations because of their mature technology and the convenience of being transported and stored (Yue *et al*., 2022). A booster shot of BNT162b2 among Malaysians was also implemented as a part of the government’s plan to increase

protection against the infection. Booster doses boost neutralising antibody levels and provide protection against SARS-CoV-2 infections and COVID-19 hospitalisations (Tartof *et al*., 2022).

Neutralising antibodies are important in monitoring the effectiveness of the vaccines against various viruses, including SARS-CoV-2. Plotkin (2010) stated neutralising antibodies or binding antibodies were discovered as an indicator of protection or a surrogate marker for vaccines against various types of viral diseases. Hence, assessing neutralising antibodies is crucial in order to observe the immune response from vaccination, which shows differences in neutralising antibody levels. Lu *et al*. (2021) stated neutralisation assays can detect neutralising antibodies against SARS-CoV-2 in quantitative terms, allowing the scientists to assess the relationship between neutralising antibody levels and disease severity. The assays can be used to evaluate the effects of monoclonal antibodies, convalescent plasma, and vaccines, as well as estimate the likelihood of re-infection in COVID-19 patients (Lu *et al*., 2021). There are various neutralisation assays available in the market to evaluate the levels of neutralising antibodies in individuals following vaccination or natural infection. The plaque reduction neutralisation test (PRNT) is the gold standard for detecting neutralising antibodies among the assays. PRNT can assess vaccine- or infection-induced humoral immunogenicity against SARS-CoV-2; however, the assay is not widely available and is costly to purchase (Habermann *et al*., 2023). Therefore, simpler and faster assays are being developed so that they can be used for both research and clinical purposes by a broader group of people.

There are two latest neutralising antibody assays available in the market that are gaining interest from many researchers, which are the GenScript cPass surrogate virus neutralisation test (sVNT) and the ImmuSAFE COVID+ assay. According to Jung *et al*. (2021), the GenScript cPass sVNT kit is the first commercial assay to be granted by Food and Drug Administration Emergency Use Authorization (FDA_EUA) that measures neutralising antibody levels qualitatively and semi-quantitatively. The cPass assay detects antibodies that prevent the interaction of two recombinant proteins, which are horseradish peroxidase conjugated receptor-binding domain (RBD-HRP) and angiotensin-converting enzyme 2 (ACE2). The ImmuSAFE COVID+ biochip test is one method for verifying the efficacy of the multiple COVID-19 vaccines administered in Malaysia (Mohamed Basyir, 2021). The test quantifies the number of protective antibodies against the virus produced by the body as a result of vaccination or natural development following infection. Therefore, a study evaluating GenScript cPass and ImmuSAFE COVID+ tests in detecting neutralising antibodies against SARS-CoV-2 can provide a better understanding of immunisation trends and health management.

1.2 Problem statement

Detecting neutralisation antibody titres is significant because the effectiveness of the vaccines can be evaluated, and immune response after natural infection or vaccination can be understood. Thus, many assays that provide measurements of neutralising antibody levels have been produced by many pharmaceutical companies. Among the available assays in the market, GenScript cPass and ImmuSAFE COVID+ tests are the current tests for detecting the presence of neutralising antibodies. However, limited data is available reporting the detection of neutralising antibodies using the GenScript

cPass and ImmuSAFE COVID+ test. The research to date has tended to focus more on analysing the neutralising antibody titres and less focus on of evaluation of the tests in terms of cost, simplicity and turnaround time. Thus, an evaluation between GenScript cPass and ImmuSAFE COVID+ assays in detecting neutralising antibody levels should be performed.

1.3 Rationale of the study

Neutralising antibody detection is an appropriate predictor and can potentially be used as a key marker for vaccine efficacy during trials (Möhlendick *et al*., 2022). There is a correlation between antibody level and protection against symptomatic COVID-19 disease. Hence, numerous assays developed in detecting neutralising antibody titres in individuals are significant. The assays that are commercially available for detecting SARS-CoV-2 neutralising antibodies include GenScript cPass sVNT and ImmuSAFE COVID+ test. Evaluating these two tests in this study can determine which test is more effective in detecting neutralising antibodies as well as faster and cost-effective. Moreover, the study can assist in evaluating the assays in terms of time, cost-effectiveness, and safety of the neutralisation assays. Besides, the study on the neutralising antibody titres between males and females among young adults can aid in developing more effective vaccination and treatment plans. Therefore, this study aimed to evaluate the current reliable neutralisation assays and better vaccination strategies can be developed based on neutralising antibody titres in genders among young adults.

1.4 Objective of the study

The general objective of the study is to evaluate GenScript cPass and ImmuSAFE COVID+ assays for detecting SARS-CoV-2 neutralising antibody titres in individuals vaccinated with CoronaVac second and BNT162b2 booster doses.

The specific objectives include:

1. To evaluate neutralising antibody titres using GenScript cPass and ImmuSAFE COVID+ in the second dose (CoronaVac) and booster dose (BNT162b2) serum samples.
2. To determine neutralising antibody titres between males and females among young adults in CoronaVac second dose and BNT162b2 booster doses serum samples.

1.5 Overview of the study

The flowchart of the study is shown in Figure 1.1.

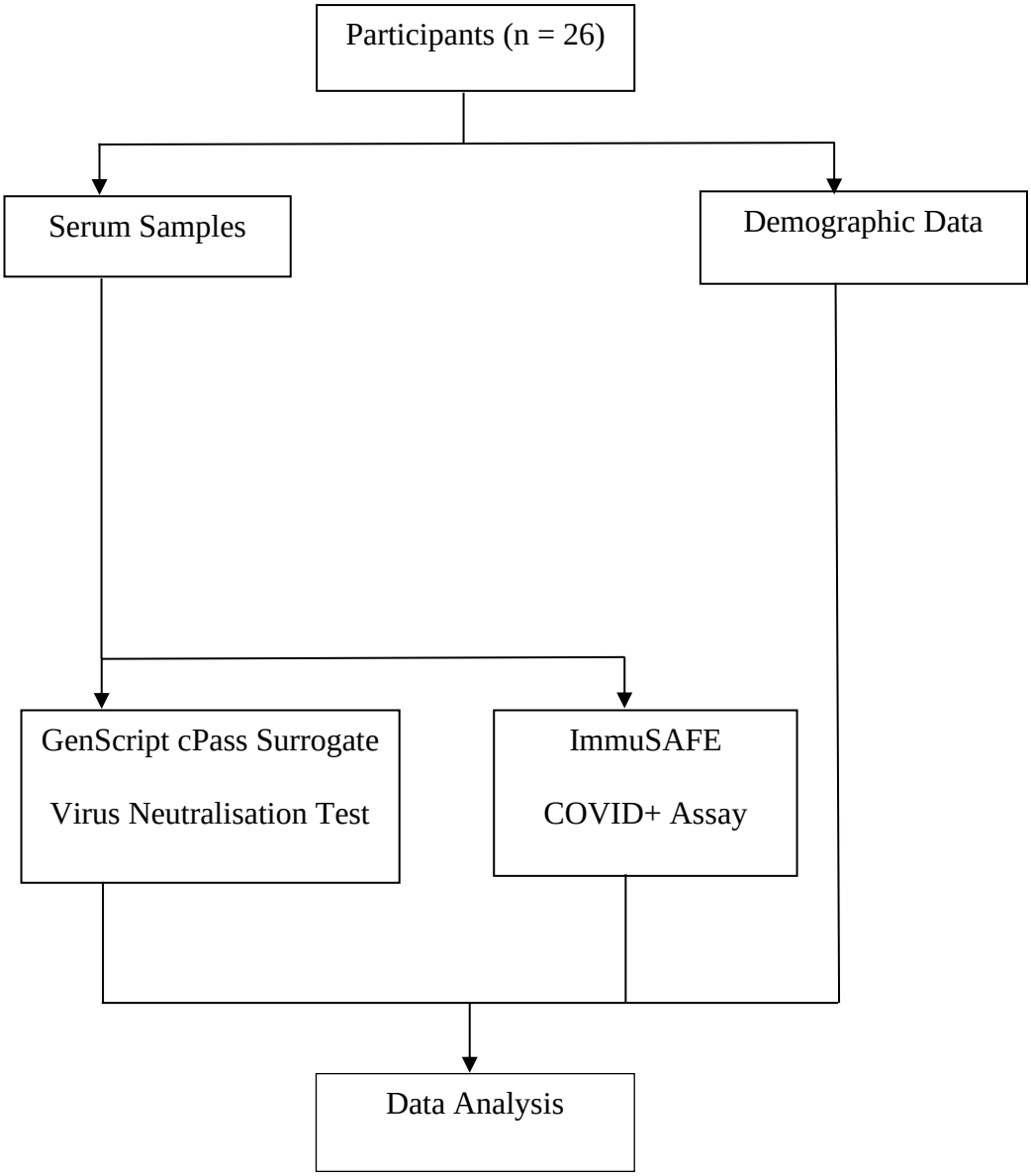

Figure 1.1 Study flowchart

CHAPTER 2

LITERATURE REVIEW

2.1 SARS-CoV-2

SARS-CoV-2 has caused the COVID-19 pandemic, which has affected many people, including population wellness and medical systems. SARS-CoV-2 is a beta-coronavirus that is remarkably similar to the human SARS-CoV virus, which was the cause of the SARS outbreak between 2002 and 2004 (Markov *et al*., 2023). The virus belongs to the species severe acute respiratory syndrome-related coronavirus, and it is the only species of the virus subgenus *Sarbecovirus*, which was discovered mainly in bats (Carabelli *et al*., 2023).

2.1.1 Structure of SARS-CoV-2

The virus is a beta-coronavirus, which is a large, spherical, enveloped, and single-stranded RNA virus that is not segmented. The replication of the approximately 30,000 nucleotide-long RNA genome of SARS-CoV-2 is carried out by the RNA-dependent RNA polymerase and an associated exoribonuclease (ExoN) enzyme (Carabelli *et al*., 2023). The protein structures form an enveloped virion that sends genomic RNA from the virus into the cell (Lamers & Haagmans, 2022). The structural proteins spike protein (S), nucleocapsid protein (N), membrane protein (M), and envelope protein (E) are found in the virion virus (Lamers & Haagmans, 2022). The SARS-CoV-2 spike prevents neutralising antibody activity. Neutralising antibodies primarily inhibit the viruses from interacting with and infecting host cells. ACE2 is a specific host cell receptor that interacts with the S1 domain of the virus to promote a conformational change in the S protein (Elengoe, 2020). S protein has both S1 and S2 domains. The

virus enters the host cell, particularly alveolar epithelial cells, through membrane fusion with the host cell membrane (Elengoe, 2020). Figure 2.1 shows the structure of SARS-Cov-2 (Lamers & Haagmans, 2022).

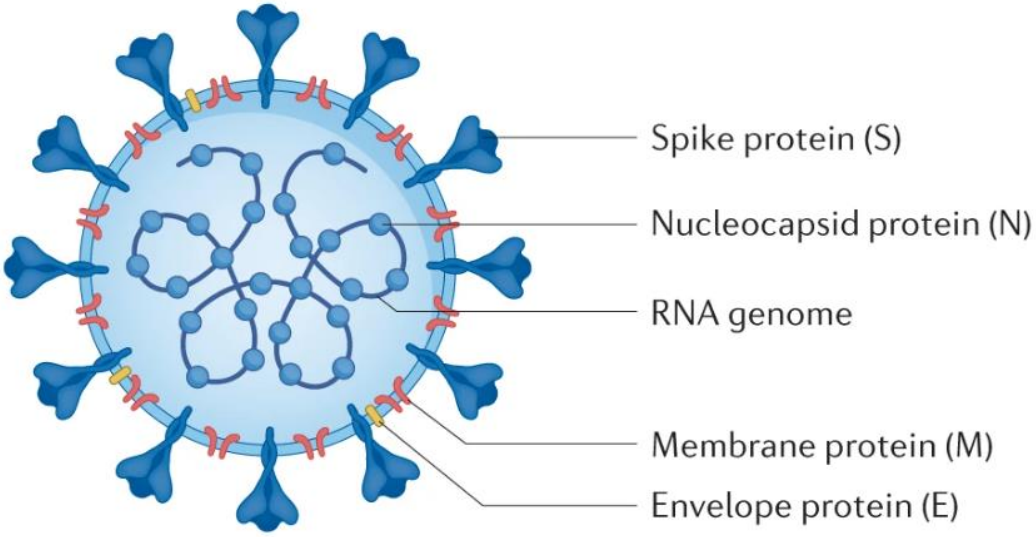

Figure 2.1 SARS-CoV-2 structure (Lamers & Haagmans, 2022)

2.1.2 Transmission of SARS-CoV-2

The mode of transmission for SARS-Cov-2 can be through animal-to-human transmission and human-to-human transmission. The modes of transmission of zoonotic COVID-19 are portrayed in Figure 2.2 (Sharma *et al*., 2021).

2.1.2 (a) Animal-to-human transmission

SARS-CoV-2 may spread from animal to human through direct contact with intermediary hosts such as snakes and bats. Bat-derived coronaviruses rarely infect humans; thus, an intermediate host is always required for SARS-CoV-2 transmission to humans (Xiao *et al*., 2020). The study by Xiao *et al*. (2020) also revealed that SARS-CoV-2-like coronaviruses were found in the wild pangolins in China and Malaya.

Therefore, the virus might have emerged from the viral recombination of pangolin and bat coronaviruses prior to spreading to humans.

2.1.2 (b) Human-to-human transmission

SARS-CoV-2 becomes airborne when someone coughs or sneezes because the primary method of human-to-human transmission involves respiratory droplets released by an infected person (Sharma *et al*., 2021). This places non-infected people at a greater chance of acquiring the disease. Another reported method of transmission involves viral contamination in hospital rooms housing COVID-19 patients (Sharma *et al*., 2021). Healthcare industries need to carefully evaluate the strategies to put into action as a way to reduce the possibility of transmission in nosocomial environments, as the virus transmits via droplets and fomites. Precautionary controls can be implemented in order to prevent potential spread during treatments along with decontaminating shared spaces, instruments, and one's own life (Sharma *et al*., 2021).

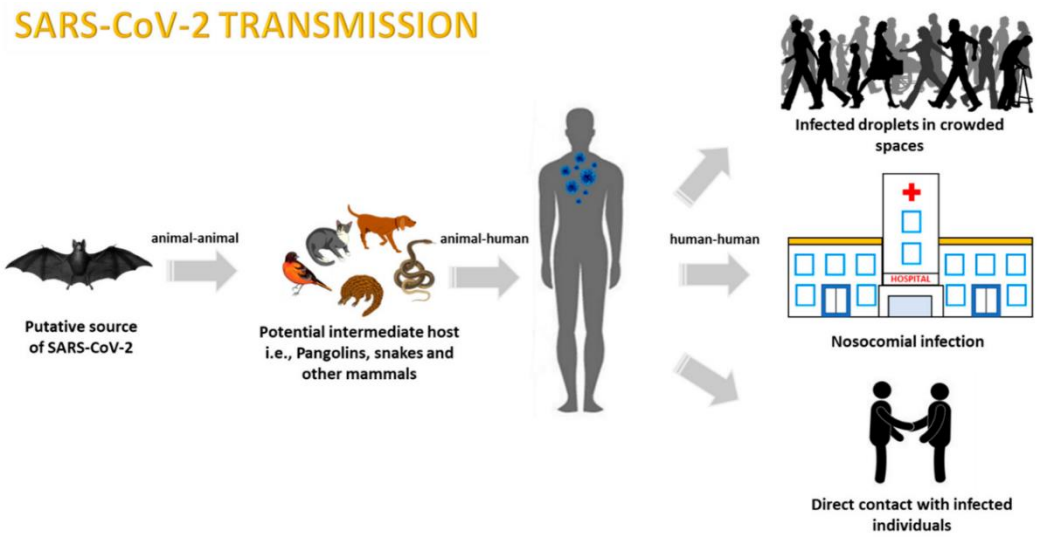

Figure 2.2 SARS-CoV-2 mode of transmission (Sharma *et al*., 2021)

2.2 Humoral immune response to COVID-19 vaccination

Upon receiving the COVID-19 vaccine, the body begins to produce a humoral immune response. A harmless strain of the SARS-CoV-2 or a component of the virus, such as the spike protein, is present in the vaccine. The vaccine is recognised as foreign by the immune system, prompting an immune response. A large number of these neutralising antibodies are produced during the humoral immune response. These antibodies can circulate in the blood vessels and attach to the spike protein, preventing it from infecting cells. Age, the use of glucocorticoids, immunosuppressive treatments, and alcohol consumption have all been associated with lower antibody titres following vaccination in a group of Japanese people (Kageyama *et al*., 2021). In the meantime, variables that predicted higher serum antibody titres included female gender, the interval between two doses of vaccines, and allergy treatment (Morales-Núñez *et al*., 2021).

2.2.1 Neutralising antibodies

The immune system creates a specific type of antibody called a neutralising antibody in response to an infection or a vaccination. Neutralising antibodies are effective at lowering pathogen titres, neutralising pathogens, and preventing infection in tissues or cells (Morales-Núñez *et al*., 2021). The antibodies can block the interaction between the receptor-binding domain (RBD) of the novel coronavirus spike protein and the ACE2 cell surface receptor (Morales-Núñez *et al*., 2021).

The first step, as shown in Figure 2.3 (Morales-Núñez *et al*., 2021), is neutralising antibodies bound to the receptor-binding protein (S) and preventing it from interacting with ACE2. In the second step, the virion contacts the cell surface receptor with its binding protein, and neutralising antibodies prevent the next step, which is binding to a co-receptor. The virion is going to fuse with the cell membrane in the third step, but neutralising antibodies attach to proteins that are not required for cell receptor binding yet exert conformational modifications that prevent virus internalisation through the cell membrane. The fourth and final step is neutralising antibodies to prevent the virion from fusing its envelope with the vesicular membrane and initiating replication. The antibody's binding to the virus prevents the virus from undergoing the conformational changes required for membrane fusion. Figure 2.3 shows a diagram of potential neutralisation mechanisms based on SARS-CoV-2 interactions with its receptors (Morales-Núñez *et al*., 2021).

The majority of studies have reported an excellent humoral response within a few days after vaccination; however, neutralising antibodies typically decline as time goes on. Memory B cells, on the other hand, can swiftly utilise more antibodies after another exposure to the virus, as T cells can attack the infected cells. Citing evidence from the previous study by Morales-Núñez *et al*. (2021), it has been established that the mRNA BNT162b2 vaccine induces robust B cell immunity and spike-specific memory B cells that continue to exist six months after vaccination, which is essential for quick response to the virus encounter. The current vaccines continue to provide clinical benefits against the majority of variants by decreasing COVID-19 disease severity. Nevertheless, the decline in neutralisation effectiveness is still an issue that needs more

research (Morales-Núñez *et al*., 2021). Therefore, neutralising antibodies can indicate the immune system's ability to fight off a specific infection.

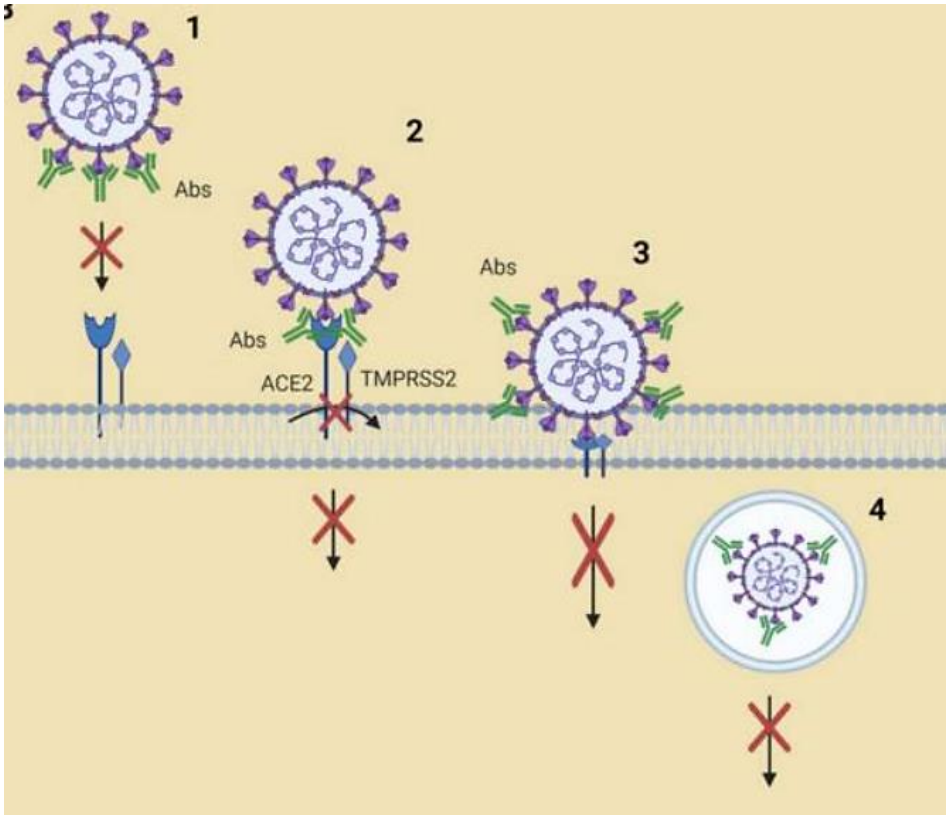

Figure 2.3 Neutralisation mechanisms between SARS-CoV-2 and its receptors (Morales-Núñez *et al*., 2021)

2.3 Virus neutralisation assays

2.3.1 Conventional virus neutralisation test

The conventional virus neutralisation test is the plaque reduction neutralisation test (PRNT). By plating the virus with susceptible cells, the PRNT allows one to test the effects of antibodies on viral infectivity (Morales-Núñez *et al*., 2021). As a result, it is regarded as the gold standard for determining antibody neutralisation capacity against SARS-CoV-2. The cells are grown in semi-solid media, which prevents the virus from spreading. Each virus that causes infection creates a localised infection known as plaque, which can be viewed and counted (Morales-Núñez *et al*., 2021). This assay has a significant disadvantage in that it is usually performed with live viruses. PRNT also requires working in a biosafety level 3 (BSL-3) laboratory under the supervision of highly skilled and knowledgeable personnel.

2.3.2 Surrogate virus neutralisation test

The surrogate virus neutralisation test detects the presence of neutralising antibodies in a sample by using a blocking enzyme-linked immunosorbent assay (ELISA) theory that imitates virus-cell interaction (Morales-Núñez *et al*., 2021). The test employs ELISA plates with immobilised human ACE2 protein (hACE2) and an RBD-HRP. hACE2 is bound in the plate, and RBD-HRP is used for detection. If neutralising antibodies are present in high concentrations, the intensity of the signal will be lower. The signal is higher if there are no neutralising antibodies because the RBD-HRP binds to the hACE2 (Morales-Núñez *et al*., 2021). The sVNT without neutralising antibody differentiation is commonly used in research. The test has a strong correlation with virus neutralisation and vaccine effectiveness against the wild-type strain regardless of the functional differences (Habermann *et al*., 2023).

2.3.2 (a) GenScript cPass SARS-CoV-2 sVNT

GenScript cPass sVNT is designed to be a rapid, precise, and large-scale alternative. In samples obtained from infected and immunised individuals, this assay displays the best linear correlation with the pseudo-virus neutralisation test (pVNT). In pVNT, a pseudo-virus expressing SARS-CoV-2 S protein fights with anti-SARS-CoV-2 neutralising antibodies, which prevent the virus from binding to hACE2 receptors on the host cell surface. The samples taken from both infected and immunised people using the test showed the best linear correlation with pVNT (Zedan *et al*., 2022). Comparing the performance of GenScript sVNT to pVNT, which exhibits high specificity and even greater sensitivity. Clinical laboratories may be able to use the GenScript cPass sVNT as a method for precisely determining protective immunity without the facilities of a BSL-3 laboratory (Morales-Núñez *et al*., 2021). This test also has the ability to determine total neutralising antibodies in an isotype-independent manner, which increases test sensitivity. This indicates that sVNT is potentially useful as an indicator of neutralising activity. Table 2.1 shows previous studies that used GenScript cPass assay in the procedure.

Table 2.1 GenScript cPass sVNT in previous studies

Year	Summary	Reference
2023	The correlation between in-house and commercial binding-specific IgG antibodies, as well as in-house and commercial SARS-CoV-2 sVNT, was assessed.	(Winichakoon *et al*., 2023)
2022	Four different assays were compared to measure SARS-CoV-2 antibodies in serial samples of 350 vaccinated individuals at three different time points after vaccination (three weeks following the first or second dose and three weeks prior to the third dose).	(Seo *et al*., 2022)
2022	The performance of three sVNTs, which include two ELISA-based tests and an automated bead-based immunoassay for the detection of neutralising antibodies against SARS-CoV-2, was reported to be excellent.	(Zedan *et al*., 2022)
2022	The performance of four quantitative commercial assays for SARS-CoV-2 spike immunoglobins was compared using the WHO's international standard.	(Zhuo *et al*., 2022)
2021	The first GenScript Biotech sVNT was tested against SARS-CoV-2 PRNT in convalescent and vaccinated people and compared to other ELISAs developed to detect neutralising antibodies.	(Hofmann *et al*., 2021)
2021	SARS-CoV-2 sVNT and the TECO SARS-CoV-2 neutralising antibody assay were compared and suitable for evaluating successful immunisation with mRNA-1273 using 93 samples from healthcare workers two to three weeks following the second vaccination with Moderna mRNA-1273 and 40 control samples from the prior to COVID virus before 2019.	(Krüttgen *et al*., 2021
2021	Six immunoassays measuring SARS-CoV-2 antibodies were compared to neutralising antibody levels in convalescent plasma to determine their efficacy and potential for prediction.	(Lamikanra *et al*., 2021)
2021	The predictive virus-neutralising activity of serum samples collected five to seven weeks after the onset of symptoms from 101 donors with previous COVID-19 infection was used to assess the diagnostic value of commonly used serological tests.	(Szabó *et al*., 2021)

2.3.3 ImmuSAFE COVID+ test

The Malaysia Automotive, Robotics and IoT Institute (MARii), Sengenics, and the Institute of Medical Research (IMR) collaboratively developed the ImmuSAFE COVID+ biochip test, which is one of the tests used to validate the efficacy of all of the vaccines used in Malaysia. The test consists of nine functionally verified domains or regions of the Nucleocapsid (N), and Spike (S) proteins of the virus expressed using Sengenics' KREX protein folding technology (Mohamed Basyir, 2021). The ImmuSAFE COVID+ test employs Sengenics patented KREX protein folding technology, which assures that viral antigens are precisely folded while maintaining all conformational and linear antibody binding sites (Farah, 2020). More than ten SARS-CoV-2 nucleocapsid and spike protein domains, including many truncated and full-length variants, are present in the test. Sengenics has created a patented technology that produces three different antibody microarrays using full-length and many truncated variants of the SARS-CoV-2 S and N proteins with multiple domains. The chip identifies various IgG, IgA, IgM, and IgG1-4 subclasses of antibodies. By determining whether the antibodies are released as the result of the vaccine itself or of a previous infection, ImmuSAFE tests are also able to evaluate the patient's response to the vaccines (Shaffaf & Ghafar-Zadeh, 2021).

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CHAPTER 3

MATERIALS AND METHODS

3.1 Ethical approval

The study was performed under approval from the Human Research Ethics Committee (HREC) of Universiti Sains Malaysia (USM), Kelantan. The human ethical approval (USM/JEPeM/21030201) is attached as Appendix B. Written informed consent was obtained from all test subjects.

3.2 Study subjects

The frozen archival serum samples were obtained from 26 healthy individuals at USM. The samples were collected two weeks after the individuals received a second CoronaVac dose. The samples from individuals vaccinated with the booster dose of BNT162b2 were also obtained after two weeks. Furthermore, demographic data such as gender, age, and history of COVID-19 past infection were gathered from the subjects. Every study subject gave their informed consent. The infection status data was recorded to ensure the samples chosen were from never-infected individuals. The samples were only taken from people who had never been infected because their neutralising antibody titres had only been increased by vaccination and not by actual infection. Thus, neutralising antibody titres were compared between the second dose of CoronaVac and booster dose of BNT162b2 vaccination using samples from never-infected individuals.

3.2.1 Sample size calculation

The sample size required for this study was calculated using GPower (version 3.1). The sample size calculation using the statistical power analysis program is shown in Appendix A. The total sample size obtained from the software was 134 considering two groups, $\alpha < 0.05$, a power of 80%, and an effect size of 0.50. However, due to the small sample size, a total of 26 samples were used in this study. The samples were divided into two groups, which were 13 samples of individuals vaccinated with the second dose of CoronaVac taken after two weeks (D2-2) and 13 samples collected two weeks after individuals received booster shot BNT162b2 (D3-2). The chosen samples for this study were from vaccinated individuals with no previous history of COVID-19 infection.

3.3 Materials

3.3.1 List of kit and consumables

The kit and consumables used in this study are listed in Table 3.1

3.3.2 List of equipment

The equipment used in this study is listed in Table 3.2.

Table 3.1 List of kit and consumables

Name	**Manufacturer**
cPass SARS-CoV-2 Neutralisation Antibody Detection Kit • Capture plate • Plate sealers • Positive control • Negative control • HRP conjugated RBD (RBD-HRP) • HRP dilution buffer • 20× wash solution • TMB solution • Stop solution	GenScript, USA
Graduated cylinder	DURAN, Germany
Laboratory bottle (1000 mL)	DURAN, Germany
Pipette tips (10 µL, 200 µL)	Axygen, USA
Conical tube (15 mL)	DURAN, Germany
96-well plate	NA
Microcentrifuge tube	NA
Reagent reservoirs	NA
Paper towels	NA
Aluminium foil	NA

Table 3.2 List of equipment

Name	**Manufacturer**
Microplate reader Infinite F50 Plus (450 nm filter)	Tecan, Switzerland
Computer	Hp, USA
Pipette (10 µL)	Sartorius, Germany
Multichannel pipette (200 µL)	Axygen, Poland
37 °C incubator	Binder, Germany
Ice maker machine	Brema, Italy
Refrigerator	Panasonic, Japan
-20 °C freezer	Haier, China
Laboratory timer	NA
Icebox	NA

3.4 Methods

3.4.1 Serum preparation

Eight mL of blood from each participant was collected at designated time points and processed into serum samples. In each 1.5 mL microcentrifuge tube, 250 µL of serum was prepared and aliquoted. Prior to use, all serum samples were kept in a freezer at a temperature of -20°C.

3.4.2 GenScript cPass test

To determine antibody titre, the GenScript cPass SARS-CoV-2 surrogate virus neutralisation test was used in the study. The overview of the test is illustrated in Figure 3.1. This assay was used on all samples following the manufacturer's instructions. The neutralisation assay plate was planned by including serum samples of CoronaVac second dose and BNT162b2 booster dose two weeks after post-vaccination. All samples were tested in triplicate, yielding 39 samples from the second dose group and 39 samples from the booster dose group. The use of triplicate samples was intended to increase the assay's reliability and ensure the result’s validity.

3.4.2 (a) Reagent preparation

Prior to use, all reagents and samples were removed from the refrigerator and given about 30 minutes to acclimatise to room temperature (20 to 25 °C). All reagents were immediately refrigerated after use. To make 1× RBD-HRP Solution, 10 µL HRP conjugated RBD was diluted in a conical tube with 10 mL HRP dilution buffer. For 1× wash solution, 40 mL of 20× wash solution was diluted with 760 mL of distilled water at a volume ratio of 1:20. When not in use, the solution was kept between 2 and 8 °C.

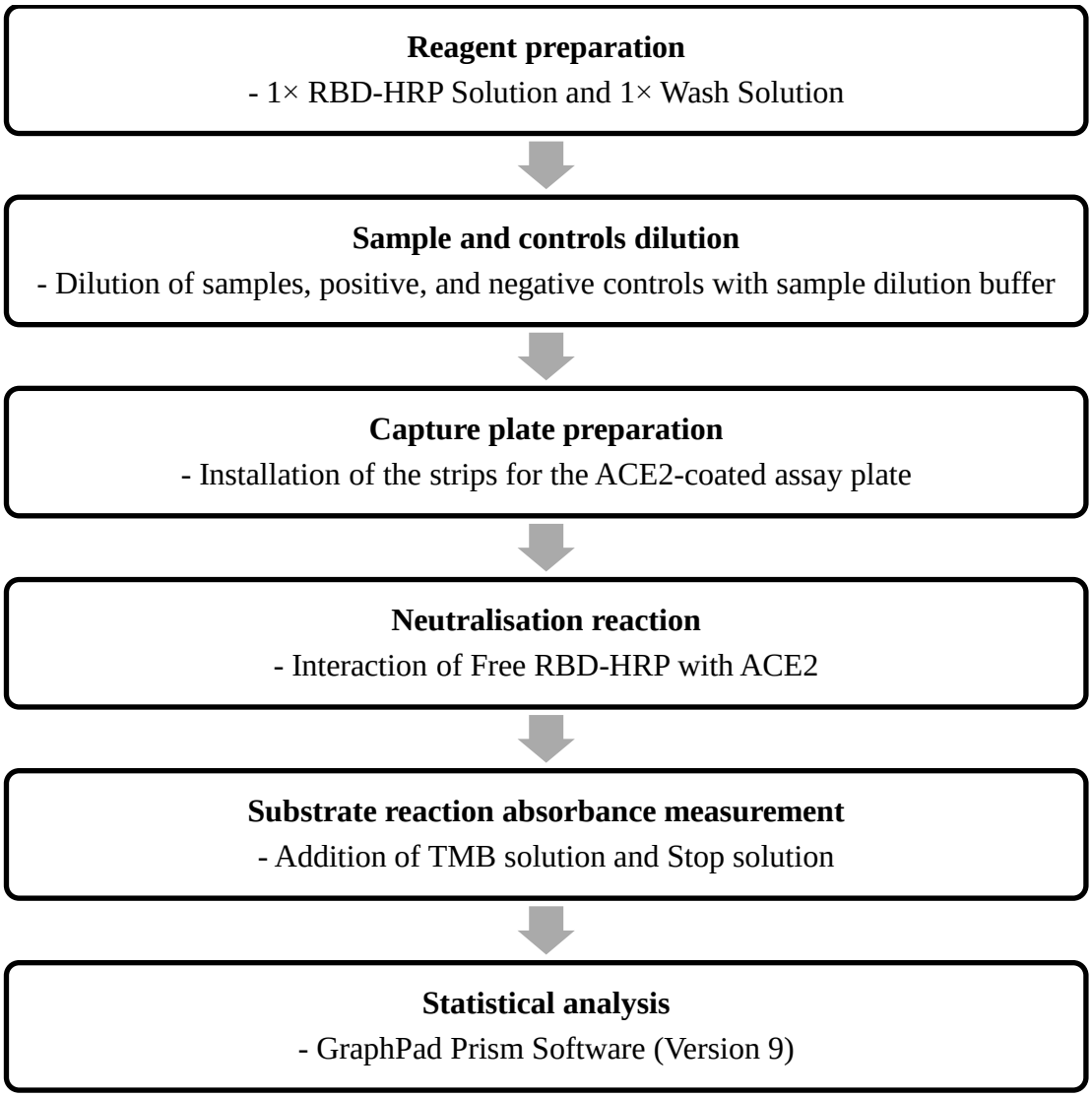

Figure 3.1 Flowchart of GenScript cPass sVNT procedure overview

3.4.2 (b) Sample and control dilution

In a 96-well dilution plate (Sample Plate), 10 µL of the test sample was diluted with 90 µL of sample dilution buffer at a volume ratio of 1:9. The positive and negative controls were then diluted at a ratio of 1:9 by combining 10 µL of control with 90 µL of sample dilution buffer in the Sample Plate. Testing was done in triplicate on both the samples and the controls. Two wells of the Sample Plate were only filled with 90 µL of sample dilution buffer.

3.4.2 (c) Capture plate preparation

To prepare the Capture Plate, the strips in the ACE2-coated assay plate were securely clicked into the plate frame. The unused strips were kept in the foil pouch and stored at a temperature of 2 to 8 °C. To prevent moisture from damaging the Capture Plate, the remaining strips were stored in a closed foil pouch.

3.4.2 (d) Neutralisation reaction

In a separate 96-well dilution plate (Neutralising Reaction Plate), 80 µL of diluted samples were mixed with 80 µL of RBD-HRP solution in a volume ratio of 1:1. Then, 80 µL of diluted controls and 80 µL of RBD-HRP solution were combined at a ratio of 1:1. The mixtures were incubated for 30 minutes at 37 °C. Following incubation, the Neutralising Reaction Plate was removed from the incubator. HRP-conjugated RBD was thoroughly resuspended with samples and controls. The corresponding wells (Capture Plate) were then filled with 100 µL of each positive control mixture, negative control mixture, and sample mixture. A plate sealer was used to tightly seal the capture plate. The plate was incubated for 15 minutes at a temperature of 37 °C. After incubation, the capture plate was washed four times with 260 µL of 1× wash solution. Following the washing steps, the plate was tapped on a paper towel in order to eliminate any remaining liquid in the wells.

3.4.2 (e) Substrate reaction absorbance measurement

The Capture Plate was filled with 100 µL of 3,3’,5,5’-Tetramethylbenzidine (TMB) solution, sealed with a plate sealer and incubated at 25 °C in the dark for 15 minutes. The timer began when the TMB was added to the first column of wells. To ensure that all reactions were incubated with the TMB solution at the exact time, 50 µL of stop