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Adaptive immune response in COVID-19 patients and innate immune modulation of SARS-CoV-2

Matefo Millicent Litabe

HEALTH SCIENCES
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Adaptive immune response in COVID-19 patients and innate immune modulation of SARS-CoV-2

Matefo Millicent Litabe

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Supervisor: Professor Felicity Burt. Division of Virology, Faculty of Health Science, University of the Free State, Bloemfontein

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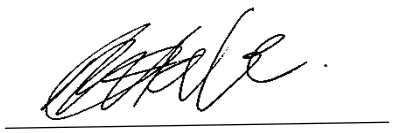
Declaration

I, Matefo Millicent Litabe declare that the Doctorate Research Thesis that I herewith submit for the Doctor of Philosophy Degree qualification in Medical Virology at the University of the Free State is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.



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My heavenly Father, who strengthens and guides me.

"Remember the Lord in everything you do, and he will show you the right way."

- **Proverbs 3:6**

Presentations and Publications

Presentations

1. Litabe MM & Burt FJ. Humoral responses against severe acute respiratory syndrome coronavirus 2 in the South African cohort. University of the Free State Health Sciences 2022 Research Faculty Forum, University of the Free State. 25-26 August 2022.
2. Litabe MM & Burt FJ. Humoral responses against severe acute respiratory syndrome coronavirus 2 in the South African cohort. 10th Annual Free State Department of Health Research Day, University of the Free State. 17 November 2022.
3. Litabe MM, Bester PA, Burt FJ. Evaluation of Cross-Neutralizing Antibody Responses to SARS-CoV-2 Variants in the South African Population following Infection and Vaccination. BRICS Young Scientist Forum and Innovator Prize 2023, Gqeberha, South Africa. 31 July - 2 August 2023.
4. Litabe MM, Bester PA, Burt FJ. Duration of immunity and cross-protection against heterologous SARS-CoV-2 variants in COVID-19 recovered individuals. University of the Free State Health Sciences 2022 Research Faculty Forum, University of the Free State. 25-26 August 2023.
5. Litabe MM, Bester PA, & Burt FJ. Unraveling the Secrets of Immunity: The COVID-19 Antibody Tale. University of the Free State, Faculty of Health Sciences, three-minute thesis competition. 18 September 2023

Publications

1. Matefo Litabe & Felicity J Burt. Severe acute respiratory syndrome coronavirus 2: Aetiologic agent of a pandemic
2. Matefo Litabe, Cloete van Vuuren, Philip Armand Bester, Dominique Goedhals, Samantha Potgieter, John Frater, Craig Thompson, Wright Daniel, Theresa Lambe, Sunetra Gupta, Mareza Brink, Danelle van Jaarsveldt, Felicity Jane Burt. Validation of laboratory developed serology assays for detection of IgG antibody to severe acute respiratory syndrome coronavirus 2 in the South African population. Journal of Virological Methods (2022) 114571.
3. Matefo Litabe & Felicity J Burt. Dynamics of IgG antibodies against severe acute respiratory syndrome coronavirus 2 in COVID-19 recovered individuals (Manuscript submitted to Health Science Reports).

4. Matefo Litabe, Philip Bester, & Felicity J Burt. Longitudinal assessment of IgG antibody responses in COVID-19 recovered patients in the Free State, South Africa (manuscript in preparation for South African Medical Journal).

5. Matefo Litabe, Philip Bester, Motlalepula Matshabisa, & Felicity J Burt. In vitro assessment of Phela and components of Phela in modulating innate immune responses against SARS-CoV-2 (Manuscript submitted to Journal of Enthopharmacology).

List of abbreviations

Abbreviation	Meaning
ABTS	2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid
ACE-2	Angiotensin-converting enzyme 2
AIM2	Absent in melanoma 2
α	Alpha
Amp	Ampicillin
ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
ATPase	Adenosine triphosphatase
β	Beta
BSL	Biosafety level
CC_{50}	Cytotoxic concentration at 50%
CLIA	Chemiluminescence immunoassay
CoV	Coronavirus
COVID-19	Coronavirus disease 2019
CPE	Cytopathic effects
CRP	C-reactive protein
Ct	Cycle threshold
d	Delta
DMEM	Dulbecco's Modified Eagle's Media
DPP4	Dipeptidyl peptidase 4
E	Envelope
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
ER	Endoplasmic reticulum
EUA	European University Association
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
γ	Gamma
GFP	Green fluorescent protein
G-CSF	Granulocyte- colony-stimulating factor
HCoV-EMC	Human coronavirus- Erasmus Medical Centre
HEKs	Human Embryonic Kidney cells
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

His	Histidine
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSD	Honest Significant Difference
ICU	Intensive care unit
IFA	Immunofluorescent assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
J&J	Johnson & Johnson
LB	Luria-Bertani
LCL	Lower control limit
LFA	Lateral flow assay
L-glut	L-glutamine
M	Membrane
MCP-1	Monocyte chemoattractant protein-1
MERS	Middle East respiratory syndrome
Mpro	Main protease
mRNA	Messenger RNA
N	Nucleocapsid
NGS	Next generation sequencing
NEAAs	Non-essential amino acids
NHLS	National Health Laboratory Services
NICD	National Institute of Communicable Diseases
NOD	Nucleotide-binding oligomerization domain
Nsps	Non-structural proteins
OD	Optical density
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PASC	Post-acute sequelae of COVID-19
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
Pen/strep	Penicillin/ streptomycin
PFU	Plaque forming units

PLP	Papain-like protease
PP	Percentage positive
PPE	Personal protective equipment
PPV	Positive predictive value
PRNT	Plaque reduction neutralization test
PRRs	Pattern recognition receptors
qRT-PCR	Real-time reverse transcriptase-polymerase chain reaction
RBD	Receptor binding domain
RdRp	RNA-dependent RNA polymerase
RIG	Retinoic acid-inducible gene 1
RNA	Ribonucleic Acid
R0	Reproduction Number
RPMI	Rosewell Park Memorial Institute
S	Spike
SA	South Africa
SAPHRA	South African Health Products Regulatory Authority
SARS-CoV	Severe acute respiratory syndrome coronavirus
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIV	Simian immunodeficiency virus
$TCID_{50}$	Tissue culture infectious dose at 50%
TG-ROC	Two-graph receiver operating characteristic
TRLs	Toll-like receptors
TNF	Tumor necrosis factor
UCL	Upper control limit
UK	United Kingdom
USA	United States of America
Vero	African green monkey kidney cells
VOC	Variant of concern
VSV	Vesicular stomatitis virus
WHO	World Health Organization

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Summary

In December 2019, a cluster of cases of atypical pneumonia were reported in Wuhan, China. All patients had a history of attending a large seafood market. Surveillance and detection methods established during the 2003 severe acute respiratory syndrome coronavirus (SARS-CoV)-1 outbreak contributed towards the identification of the virus as a novel coronavirus (CoV). The virus was later named SARS-CoV-2 and identified as the causative agent of Coronavirus Disease 2019 (COVID-19). Despite massive attempts to contain the virus in China, the cases rapidly increased and spread globally, and the World Health Organization (WHO) declared COVID-19 a Public Health Emergency of International Concern on 30 January 2020 and characterized the outbreak as a pandemic on 11 March 2020. Assessing immunoglobulin (Ig)-G and neutralizing antibodies is essential to comprehensively evaluate the efficacy and duration of immunity conferred by natural infection and the COVID-19 vaccines. Effectively determining SARS-CoV-2 seroprevalence within a population is important as it helps to improve our understanding of virus circulation dynamics, identify individuals at risk of infection, and the extent of virus exposure in the community. Therefore, this study investigated the duration and kinetics of adaptive immunity, particularly the persistence of IgG and neutralizing antibodies, in patients who recovered from SARS-CoV-2 in the Free State, South Africa.

Commercial assays are expensive, and hence two anti-spike (S) inhouse assays, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA), were developed and validated for detection of anti-S IgG. A total of 89 serum samples were collected from COVID-19 PCR-confirmed patients between 2-94 days post-symptom onset to validate the assay. A 100 prepandemic samples were used as a negative control panel to determine the cutoff of the assay. A cutoff value of 30% was considered accurate to differentiate between negative and positive samples using a two-graph receiver operating characteristic (TG-ROC). The assays exhibited a sensitivity of 100% for ELISA and 98.8% for IFA when testing samples collected more than one week after the onset of illness. The positive predictive values (ppv) were 92.1% for ELISA and 91.0% for IFA on PCR-confirmed positive samples. The assays were also compared to a commercially available SAPRHA-approved assay, where the ELISA showed a higher ppv of 95.8 %, while the Roche assay was 89.6 %, and the commercial lateral flow was 93.9 %. The two in-house assays also detected IgG

antibodies in samples collected from waves in which new variants were circulating, showing that despite mutations of the SARS-CoV-2 S protein, the assay was still sensitive to detect IgG antibodies in circulating variants. This indicates that these assays could be used for surveillance of the South African population.

To investigate the duration of anti-S IgG and neutralizing antibodies against SARS-CoV-2, 100 individuals with previously confirmed COVID-19 infection were recruited for the study. The cohort included 64/100 vaccinated and 36/100 unvaccinated individuals. Initial samples were collected between March 2021 and January 2022, with confirmed infection between June 2020 and December 2021. Follow-up samples were collected from 82/100 in 2022 and 62/100 in 2023 of the initial cohort. Samples were tested for anti-S IgG antibodies and neutralizing antibodies against Ancestral strain, Delta, and Omicron variants. A total of 95/100 baseline samples tested positive for anti-S IgG and 79/82 and 53/62 for follow-up samples. A total of 99/100, 78/100, and 72/100 baseline samples tested positive for neutralizing antibodies against the Ancestral strain, Delta, and Omicron variants. A total of 80/82, 63/82, 77/82 in 2022 and 53/62, 50/62, 56/62 in 2023 tested positive for neutralizing antibodies against the Ancestral, Delta, and Omicron variants, respectively. Samples were grouped based on the time (days) the samples were collected post-onset of illness. Results showed that IgG antibodies were significantly higher directly after infection, between 1-180 days, then gradually declined significantly with time. Neutralizing antibody titers against the Ancestral strain and Delta variant were significantly higher early after infection, between 1-180 days, remained relatively stable for an extended period, and then waned gradually before declining significantly. In contrast, although not significant, neutralizing antibodies against the Omicron variant increased with time, but this may be because samples were collected when the Omicron variant was still prevalent. Results also showed that vaccinated individuals had significantly higher antibody titers than unvaccinated, highlighting the importance of continued vaccination. The study shows the longevity of antibodies against SARS-CoV-2, as most individuals still had detectable titers at least 24 months post-acute infection, possibly boosted by vaccination or reinfection. The study also shows that the dynamics of antibodies vary among individuals; most individuals display declining antibody titers with time, and a smaller proportion maintain stable antibodies or a fluctuation of antibodies over time.

Traditional medicinal plants have been proposed as promising, cost-effective treatments for SARS-CoV-2, with studies showing the potential to induce protection against different viral infections. The study also investigated the potential of Phela, a traditional medicine prepared from the extracts of four South African plants, and the individual components to modulate the release of cytokines in SARS-CoV-2 Omicron-infected mammalian cells and to investigate the influence of the plant extracts on viral replication. Cells were treated with the plant extracts before or after infection with SARS-CoV-2. Subsequently, cell culture media was collected at 12, 24, 48, and 72 hours post-infection and tested for virus replication and levels of Interleukin (IL)-1β, IL-2Rα, IL-6, IL-10, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ cytokines. There was no statistically significant difference in viral load between infected cells treated with plant extracts compared to infected and untreated cells, showing that the plant extracts may have little to no effect on virus replication. Treatment with plant extracts resulted in significantly lower release of IL-1β, IL-2Rα, and TNF-α, with better response post-treatment than pretreatment, showing that the plant extracts may have the potential to manage cytokine storms and be a potential source of treatment for SARS-CoV-2.

Chapter 1

Orientation, rationale, and aims of the study

Introduction

In December 2019, a cluster of cases of atypical pneumonia were reported in Wuhan, China. All patients had a history of attending a large seafood market. Surveillance and detection methods established during the 2003 severe acute respiratory syndrome coronavirus (SARS-CoV)-1 outbreak contributed to identifying the virus as a novel coronavirus (CoV). The virus was later named SARS-CoV-2 and identified as the causative agent of Coronavirus Disease of 2019 (COVID-19). Despite massive attempts to contain the virus in China, the cases rapidly increased and spread globally, and the World Health Organization (WHO) declared COVID-19 a Public Health Emergency of International Concern on 30 January 2020 and characterized the outbreak as a pandemic on 11 March 2020 (WHO, 2020). SARS-CoV-2 is efficiently transmitted from person to person and thus was able to spread rapidly across the world, resulting in widespread global morbidity and mortality.

Our understanding of SARS-CoV-2 immunity has evolved since 2020, and much progress has been made in understanding immune correlates of protection. Studies have shown that both humoral and cellular immunity play a protective role (Feng et al. 2021). The immune system is divided into the innate and the adaptive immune responses, which are linked, and each plays distinct yet interconnected roles. The innate immune system acts as the body's first line of defense, providing immediate but general protection. This includes creating an antiviral state in the local tissue environment and recruiting effector cells (Goldstein and Scull 2022). It restricts replication within the infected cells and primes the adaptive immune response. The adaptive immune system is made up of B cells, $CD4^+$ and $CD8^+$ T cells. The B cells produce antibodies, $CD4^+$ helper T cells are involved in helper and effector functionalities, and cytotoxic $CD8^+$ T cells kill infected cells. The adaptive immune response plays an essential role in the control and clearance of viruses, and immune memory is essential for vaccine-making success. Thus, it is crucial to understand the adaptive immune response fully. Previous studies have found a robust humoral response in SARS-CoV patients with high neutralizing antibody titers during

convalescence (Ho et al. 2005). It is important to understand how long these antibodies last and the level of protection they offer against re-infection and severe disease. Monitoring the persistence of antibodies is important in assessing both natural and vaccine-induced immunity, as it helps determine the possibility of re-infection and the potential of attaining herd immunity. This knowledge will help guide vaccine strategies, helping to determine the optimal time for boosters. Additionally, monitoring antibody dynamics provides insight into the impact of emerging variants, as some evade immune responses, leading to breakthrough infections, emphasizing the need for continued research on immune responses.

Patients with COVID-19 also present with increased release of proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin 1beta (IL-1β), and IL-6, which play a role in virus clearance. However, dysregulated release of these cytokines results in a cytokine storm, which enhances disease severity. IL-1β and IL-6 have been associated with disease severity, and IL-6 is used as a marker for severe SARS-CoV-2 (Chen et al., 2020b). High levels of IL-6 have been associated with disease severity, organ failure, and death (Lavillegrand et al., 2021). Effective antivirals are needed to modulate innate immunity, and previous studies have focused on repurposing previous antivirals, including human immunodeficiency virus (HIV) antivirals such as HIV-1 protease inhibitors lopinavir (Mahdi et al., 2020), the hepatitis C virus protease inhibitor danoprevir (Chen et al., 2020a), and the influenza antiviral favipiravir (Kaptein et al. 2020). Repurposing of these drugs was thought to be a rapid route toward obtaining treatment. Additionally, traditional medicine was proposed as an affordable alternative treatment for SARS-CoV-2. Countries have used different traditional plants and plant extracts to treat SARS-CoV-2 (Nie et al., 2021; Tang et al., 2021; Flórez-álvarez et al., 2022). A study has proposed that traditional medicines strengthen healing by modulating the host's immune responses (Xu et al., 2023). Thus, studying innate immune modulation to SARS-CoV-2 is essential for identifying potential drug targets to prevent or mitigate cytokine storms.

Problem Identification

CoVs are an important public health problem leading to outbreaks of severe disease such as the 2002 SARS-CoV (Tsang et al., 2003), 2012 Middle East Respiratory

coronavirus (MERS-CoV) (MMWR, 2013), and the recent SARS-CoV-2 (Wu et al., 2020) outbreak with significant fatalities and substantial economic loss. SARS-CoV-2 was newly identified as a highly contagious virus that emerged in 2019 from an unknown reservoir (Wu et al. 2020). The emergence of the SARS-CoV-2 and the subsequent global pandemic caused by COVID-19 has led to unprecedented challenges in public health and healthcare systems worldwide. The virus quickly spread to 217 countries, affecting over 770 million individuals and resulting in over 6.95 million deaths (https://data.who.int/dashboards/covid19/cases). The clinical significance of SARS-CoV-2 is highly variable, ranging from self-limiting symptoms to pneumonia (Wang, et al., 2020). The dynamics of the immune response, particularly the production and persistence of IgG and neutralizing antibodies, are not yet fully understood over an extended period. Assessing IgG and neutralizing antibodies is essential to comprehensively evaluate the efficacy and duration of immunity conferred by natural infection and the COVID-19 vaccines. Previous studies show that SARS-CoV humoral responses play a crucial role in protecting against the virus, and neutralizing antibodies found in patients and animals block virus entry by binding to the spike glycoprotein (Temperton et al., 2005). Therefore, it is vital to understand adaptive immune responses in patients who have recovered from COVID-19, the duration of immunity, and the possibility of immunity being able to protect against reinfection. This study investigated the duration and kinetics of adaptive immunity in patients who recovered from SARS-CoV-2. Blood was collected from COVID-19-recovered individuals over three years to characterize the duration of IgG and neutralizing antibody responses in a cohort of patients in the Free State, South Africa. The study considered the cross-reactivity and cross-neutralization against emerging variants of concern and the role of vaccines in maintaining detectable antibody responses.

The severity of disease is frequently enhanced by a cytokine storm in individuals (Qin et al., 2020). Treatment that upregulates or downregulates responses that are key to the immunopathogenesis of the infection could play a role in treatment. The use of indigenous plants has been proposed for various viral infections (Mehrbod et al., 2018; Mehrbod et al., 2019). Hence, this study investigated the influence of selected potential immune modulators to determine whether they can influence cytokine responses against SARS-CoV-2 using mammalian cell culture. Understanding the kinetics of innate immune modulation and how these responses are influenced by the

timing and dosage of immune modulators could place us a step closer to designing precise treatment protocols.

Aims

To investigate SARS-CoV-2 antibody kinetics and determine the duration of immunity in previously COVID-19-infected patients.

To determine the effects of immune modulators on the innate immune system against SARS-CoV-2.

Objectives

1. To develop and validate in-house assays for detecting SARS-CoV-2 IgG antibodies.
2. To characterize the humoral immunity in COVID-19 patients in the South African population.
3. To evaluate possible immune modulation activities of various immune modulators against SARS-CoV-2.

Structure of the thesis

The thesis is presented as articles that are published or will be submitted for publication to various peer-reviewed scientific journals, according to the guidelines for submission of a thesis in article format from the University of the Free State. Chapter 1 is the introduction, problem statement, and study aims and objectives. The first article presented in Chapter 2 is a review article of SARS-CoV-2. The next article reported in Chapter 3 is the development and validation of two in-house serological assays for detecting IgG antibodies in serum samples of SARS-CoV-2 survivors. Two assays, ELISA and IFA, were developed based on mammalian-expressed SARS-CoV-2 spike protein and validated using samples from survivors in the South African population. The manuscript has been published in the Journal of Virological Methods (Matefo et al. 2022). Article reported in Chapter 4 investigates the duration of anti-S IgG antibodies and neutralizing antibodies against SARS-CoV-2 Ancestral strain, Delta, and Omicron variants in COVID-19-recovered patients in Free State, South Africa. A total of 244 samples were collected from 100 patients with previously confirmed SARS-CoV-2 infection recruited for the study between March 2021 and March 2023 to investigate the duration and kinetics of COVID-19 antibody responses over two years. Article reported in Chapter 5 investigates the dynamics of anti-S IgG

antibodies in COVID-19-recovered patients. Three sequential samples were collected from 62 patients between July 2021 and March 2023 and serially diluted fourfold to determine the endpoint titer for each individual. Article reported in Chapter 6 examines the ability of plant extracts to modulate cytokine responses. These plant extracts are currently under development for use as immune boosters in South Africa. Phela, a traditional medicinal plant, and the four components that make up Phela were investigated *in vitro* to determine their immune-modulatory effects against the SARS-CoV-2 Omicron variant *in vitro* by analyzing the changes in viral RNA load and cytokine levels related to cytokine storm in cells infected with the virus and treated with individual and combined plant extracts. Chapter 7 gives an overall conclusion and future perspectives for further research.

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Chapter 2

Severe acute respiratory syndrome coronavirus 2: Aetiologic agent of a pandemic

Matefo Litabe[1], Felicity Burt [1,2]

1. Division of Virology, Faculty of Health Sciences, University of the Free State, Bloemfontein, South Africa
2. Division of Virology, National Health Laboratory Service, Bloemfontein, South Africa

2.1. Introduction and History

In December 2019, the first cases of atypical pneumonia were reported in Wuhan, China. Sequence analysis identified the aetiologic agent as a novel beta (β) coronavirus (CoV), closely related to severe acute respiratory syndrome coronavirus (SARS-CoV) (Lu et al., 2020). Previous zoonotic CoV outbreaks in humans have been described. In November 2002, SARS-CoV emerged in the Guangdong province of China, resulting in more than 8000 cases and more than 700 fatalities, spreading to 29 countries. It likely emerged in wet animal markets in China, spreading to humans from an intermediate host, Asian palm civets, and originating in cave-dwelling bats in Yunnan (Guan et al., 2003; Li et al., 2005). In 2012, the Middle East Respiratory Syndrome (MERS)-CoV emerged in Saudi Arabia and has spread to 27 countries, resulting in more than 2000 cases with 858 fatalities (Memish et al., 2020). Multiple sporadic outbreaks of MERS-CoV have been described in contrast with SARS-CoV and SARS-CoV-2, which likely resulted from a single introduction from an animal source to humans.

By January 2, 2020, 41 patients with laboratory-confirmed cases of COVID-19 were identified (Huang et al., 2020; Lu and Stratton, 2020). Most patients were presumed to have contracted the virus in a hospital setting. Therefore, it was decided that the novel CoV was not a super-spreading virus. Towards the end of January 2020, more than 7000 cases of COVID-19 were reported in China, and the virus quickly spread worldwide, with confirmed cases in Thailand, Vietnam, Japan, the United States of America (USA), Australia, and the United Arab Emirates (Bassetti et al., 2020). As the virus spread globally, the World Health Organization (WHO) declared a public health emergency. The pandemic resulted in a worldwide lockdown to manage the spread of

the disease. Currently, over 774 million COVID-19 cases and 6.95 million deaths have been reported (https://covid19.who.int/). SARS-CoV-2 has spread to 217 countries and territories worldwide, with case fatality rates of about 0.90%.

In an attempt to control the pandemic, vaccines were introduced worldwide. The first vaccine approved by the Food and Drug Administration (FDA) was the BNT162b2 by Pfizer-BioNTech, followed by others, including Johnson and Johnson, AstraZenica, Moderna, Novavax, and most recently, Spikevax. The pandemic was declared over by the WHO on May 5, 2023 as the number of weekly positive cases reduced significantly due to the widespread vaccination, availability of better treatment plans, and increased population immunity due to previous infections.

2.2. Virus classification and genome

CoVs belong to the *Coronaviridae* family, in the order Nidovirales. They are classified into four genera, including alpha (α), β, delta (d), and gamma (γ) CoVs based on serological and genetic properties (Woo et al., 2012). The β-CoV genus is further divided into five lineages. To date, there are seven CoVs known to cause human infections: two α-CoV (NL63 and 229E) and five β-CoVs (SARS-CoV, OC43, MERS-CoV, HKU1, and SARS-CoV-2). The four common CoVs (OC43, HKU1, NL63, and 229E) cause mild upper respiratory infections (Woo et al., 2012). In immunocompromised individuals, infection with these viruses can result in lower respiratory infection (Theamboonlers et al., 2007; Arden et al., 2005). In contrast, SARS-CoV, MERS-CoV, and SARS-CoV-2 infections result in more severe disease, and these viruses are capable of causing epidemics with significant morbidity and mortality. SARS-CoV-2 shares at least 80% sequence identity with SARS-CoV and about 50% with MERS-CoV (Lu et al., 2020; Zhou et al., 2020).

CoVs are single-stranded positive-sense ribonucleic acid (RNA) viruses. The virus appears crown-like under an electron microscope due to spike (S) glycoprotein which appears like a spikey projection (Melanthota et al., 2020). The CoV genome size varies between 26-30 kb with a 5' cap structure and 3' polyadenylation, the largest of RNA viruses (Wu et al., 2020). It consists of at least fourteen open reading frames (ORF) that encode two polyproteins that are cleaved into sixteen non-structural proteins (nsps) required for viral synthesis, eight accessory proteins, and four structural

proteins involved in viral assembly (Marra et al., 2003). Figure 1 shows the genomic organization of CoVs. The replicase locus is encoded within the 5'-terminal, and the structural proteins are encoded in the 3'-terminal of the genome. The genome of CoVs encodes four structural proteins, including the S glycoprotein, the nucleoprotein (N), the E, and the membrane (M) protein. The S protein makes up the spikes on the surface of the virus and is required for virus attachment and fusion. The N protein complexes with the genomic RNA to form the capsid structure embedded within the viral envelope. The E protein is involved in virus assembly and pathogenesis. The M protein shapes the virion and binds to the nucleocapsid (Chen et al., 2020).

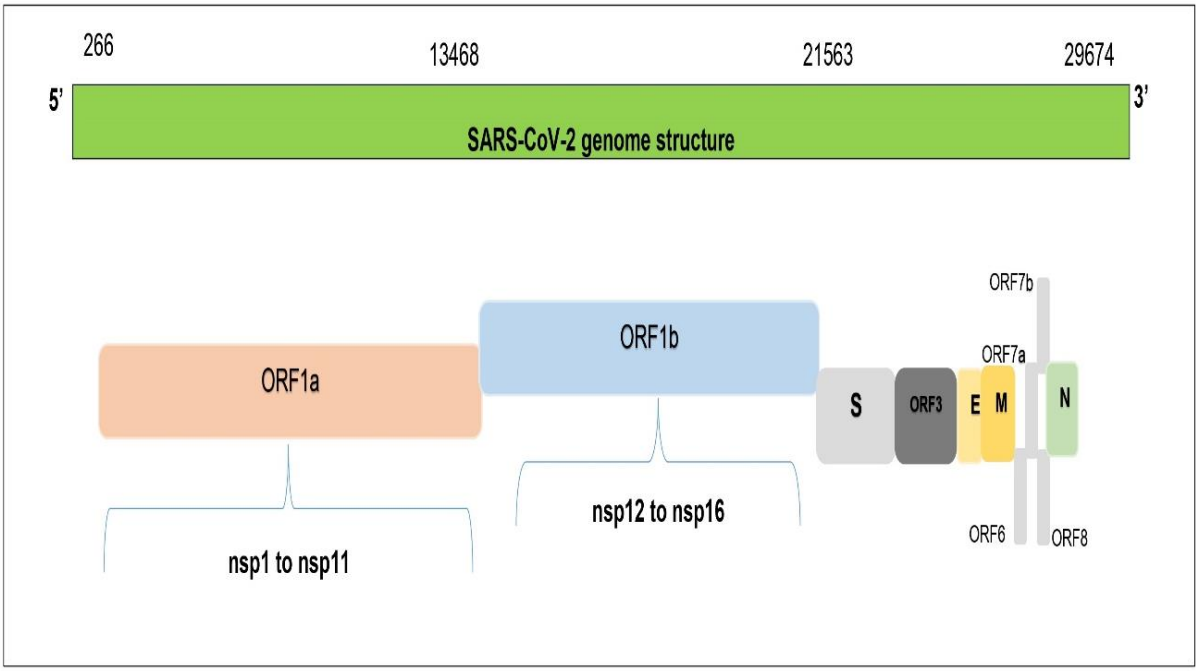

Figure 1: Genome organization of SARS-CoV-2. The genome of CoVs is about 30 kb with a 5' cap structure and 3' polyadenylation. The genome encodes two large protein, ORF1a and ORF1b, which encode 16 non-structural proteins (ns1 to ns16). The structural genes encode the structural proteins, spike (S), envelope (E), membrane (M), and nucleocapsid (N) (Modified from Zhang et al., 2021)

2.3. Replication

Following fusion, the CoV genome is released into the cytosol, where it is translated into two ORFs, 1a and 1b. The polyproteins are then cleaved by the Main protease (Mpro) and the papain-like protease (PLP) into individual proteins required for viral replication, including RNA-dependent RNA polymerase (RdRp), helicase, and

adenosine triphosphatase (ATPase). The proteins are responsible for virus replication and the generation of transcripts involved in synthesizing structural proteins. The parental genomic RNA serves as a template for synthesizing the full-length and subgenomic negative-strand RNA. Subgenomic RNA synthesis occurs from the discontinuous sequence that joins leader RNA sequences encoded at the 5' terminal of the genome to the body sequences of each genomic RNA. The eight different subgenomic negative strands serve as a template for synthesizing similar sub-genomic mRNA (Marra et al., 2003). The replicase, accessory proteins, and structural proteins are synthesized during this replication phase. The M and the E proteins are then localized to the Golgi apparatus and transversed to the endoplasmic reticulum (ER)/ Golgi intermediate compartment, where budding occurs. The S, M, N, and E proteins bind to the genomic RNA and assemble into a virion (Chen et al., 2020). A summary of the replication cycle is depicted in Figure 2 below.

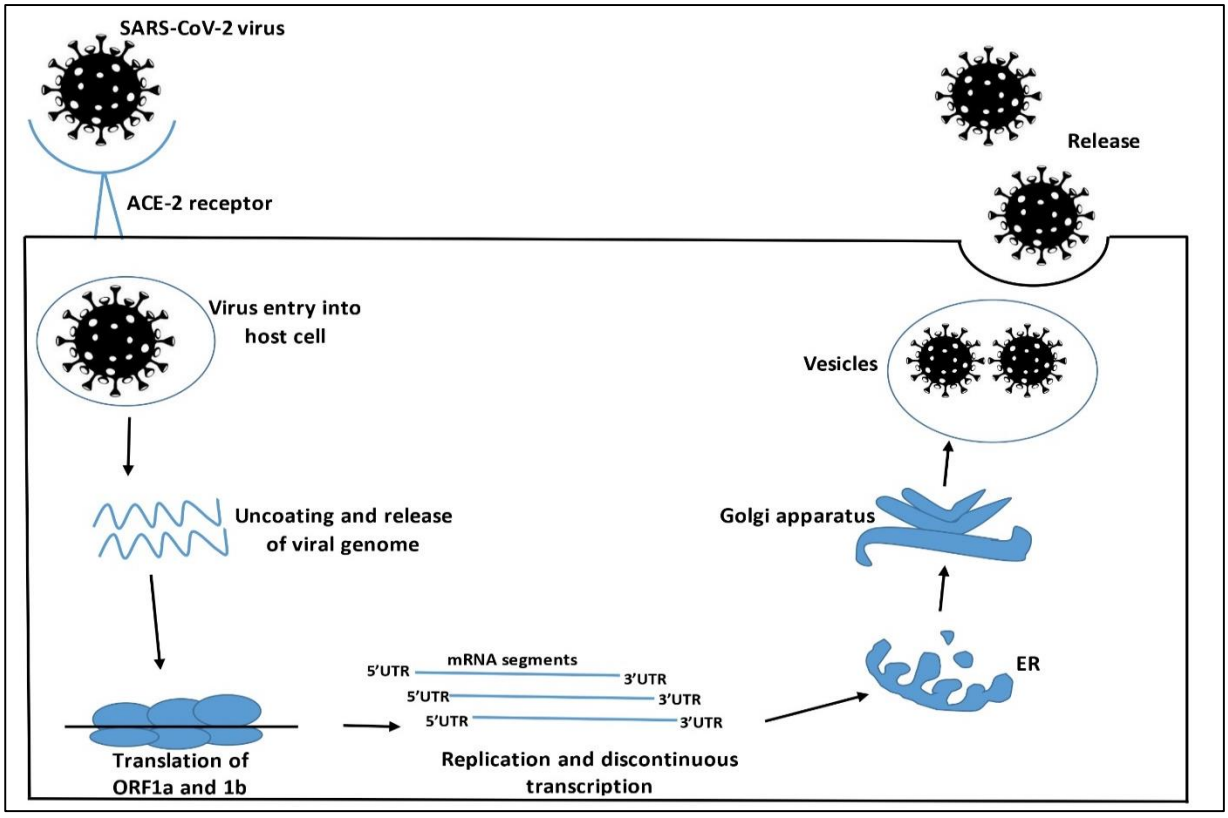

Figure 2: Replication cycle of SARS-CoV-2. The virus enters through the respiratory tract by binding to the ACE-2 receptor. After uncoating and release of the viral genome, the replicase is translated from the genomic RNA. Replication and discontinuous transcription occurs, inserted into the endoplasmic reticulum (ER), and move to the Golgi apparatus. Formation of mature virion occurs and the virus released from the cell.

2.4. Pathogenesis

CoVs enter the host cell via the respiratory tract. The virus binds to cells expressing the angiotensin-converting enzyme 2 (ACE-2) receptor, including the alveolar epithelial cells, vascular endothelial cells, and alveolar macrophages for SARS-CoV and SARS-CoV-2 (Li et al., 2003; Lu et al., 2020). For MERS-CoV, infection occurs by virus binding to dipeptidyl peptidase (DPP4), also known as CD28 (Doremalen et al., 2014). Studies have shown that the interaction of the MERS-CoV S protein with the DPP4 receptor facilitates entry and induces immune suppression in infected patients, allowing viral replication and spread (Doremalen et al., 2014). The incubation of SARS-CoV-2 Ancestral strain ranges from 2-12 days, during which the virus is transmissible (Du et al., 2022). However, the incubation period for other variants of concern (VOC) is shorter (Du et al., 2022). The incubation for the Delta variant is estimated to range between 2-6 days. The incubation period of the Omicron variant appears to be shorter than that of the Delta, ranging between 2-5 days. Active replication and release of the virus in the lung cells leads to non-specific symptoms. The most common symptoms include coughing, headache, fatigue, sore throat, and shortness of breath, which can progress to acute respiratory distress, pneumonia, renal failure, or death (Chakraborty et al., 2020). The ACE-2 receptors are found in various tissues and organs, including the intestines, endothelial cells in the kidneys, and blood vessels (Albini et al., 2020). This may explain the diverse symptoms during COVID-19 infection. Post-infection inflammatory responses attract T-cells to the site of infection, where they kill infected cells, preventing the spread of the virus and leading to recovery in most patients (Tay et al., 2020). Greater COVID-19 morbidity, intensive care unit (ICU) admissions, and acute respiratory distress syndrome (ARDS) progression are seen mainly in the elderly population relative to the younger, and men had a higher risk of COVID-19 death than women (Xu et al., 2020; Yanez et al., 2020). Other studies also reported different persistent symptoms with different durations and frequencies among COVID-19 survivors termed long COVID-19 or post-acute sequelae of COVID-19 (PASC) affecting survivors of COVID-19 of all disease severity (Dennis et al., 2021; Saberian et al., 2022). These symptoms can vary widely, including fatigue, chest pains, digestive issues, shortness of breath, and joint pain (Galal et al., 2021). The cause of persistent symptoms is still unknown. However, it has been suggested that it may be due to residual viral particles, an overactive immune

response, or autoimmune reactions (Dani et al., 2021). Individuals with mild initial symptoms can also experience long-term complications.

2.4.1. Innate immunity

Early control of viral replication via innate immunity limits viral spread during the initial phase of infection. Innate immunity also plays a critical role in shaping the adaptive immune response. Macrophages, monocytes, and dendritic cells function as the first line of defense in innate immunity. These cells have pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), inducing a direct antiviral response through the secretion of various mediators. The primary PRRs include absent in melanoma 2 (AIM2)-like receptors, C-type lectin receptors, retinoic acid-inducible gene I (RIG-I)-like receptors, Toll-like receptors (TLRs), and the nucleotide-binding oligomerization domain (NOD)-like receptors (Kanneganti, 2020). These PRRs induce innate immune cells to produce inflammatory mediators that induce antiviral protection and cell death to clear infected cells. Patients with COVID-19 present with increased levels of proinflammatory cytokines, including interferon (IFN)-β and γ, interleukins (IL)-6, IL-1β, tumor necrosis factor-alpha (TNF)-α, and monocyte chemoattractant protein-1 (MCP-1) (Hadjadj et al., 2020; (Huang et al., 2020). These cytokines play a role in the clearance of viral infection. However, over-secretion may also contribute to innate pathology and viral infectivity, with the dysregulated release of proinflammatory cytokines results in a cytokine storm. Increased levels of IL-1β and IL-18 in plasma correlate with disease severity and mortality in patients with COVID-19 (Qin et al., 2020). Severe disease outcome is also characterized by a slow decline in viral load and increased secretion of TNF, IFN-α, and IFN-γ (Lucas et al., 2020). Severe cases of SARS-CoV-2 present with high levels of granulocyte-colony-stimulating factor (G-CSF) and IL-2, which are suggested to increase the severity of the disease (Huang et al., 2020). Similar results are seen in SARS-CoV and MERS-CoV infection, where infection results in a cytokine storm (Nicholls et al., 2003;Wong et al., 2004). Increased neutrophils and decreased lymphocytes have been associated with disease severity and death (Wong et al., 2004; Tao et al., 2020). These studies indicate that cytokines may play a role in disease progression and severity. Type I IFN is essential as a first-line defense in innate immunity, controlling viral replication and induction of effective adaptive immune response. Studies indicate that MERS-CoV has evolved strategies to manipulate

innate immunity by blocking the IFN pathway, which may explain the high fatality rates associated with MERS infections (Niemeyer et al., 2013). A study on SARS-CoV-2 showed that patients with impaired type I IFN response (no IFN-β and low IFN-α production and activity) had persistent blood viral load and an exacerbated inflammatory response (Hadjadj et al., 2020), suggesting that type I IFN deficiency in the blood could be a hallmark of severe COVID-19.

2.4.2. Adaptive immunity

The adaptive immune response consists of three major cell types: B cells, $CD4^+$ T helper cells, and cytotoxic $CD8^+$ T cells, which all play a crucial role in the defense against CoVs. A natural SARS-CoV-2 infection and vaccination results in both cellular and humoral immunity, where neutralizing antibodies and T-cells are considered a correlate of protection (Hvidt et al., 2023)(Pitiriga et al., 2023). Previous studies have found a robust humoral response in SARS-CoV patients with high neutralizing antibody titers during convalescence (Ho et al., 2005). Patients with a longer duration of SARS-CoV infection had lower neutralizing antibodies than those with a quick recovery (Ho et al., 2005). The S protein is the main target for humoral immune responses (Faustini et al., 2021). Age, sex, symptomatic infection, and disease severity play a role in determining SARS-CoV-2 immunoglobulin (Ig) G and neutralizing activity (Parker et al., 2023). Older individuals, males, hospitalized patients, and patients with pre-existing conditions demonstrate more potent neutralizing antibodies (Vanshylla et al., 2021). Following infection, SARS-CoV-2 IgG antibodies significantly decline in the first few months post-infection before stabilizing (Turner et al., 2021; Vanshylla et al., 2021). Measurement of anti-S antibodies over ten months showed a half-life of 34.9 weeks, with anti-S IgG levels decreasing between 7-18 weeks post-symptom onset and remaining relatively stable for up to 10 months (Vanshylla et al., 2021). Convalescent individuals also induce S-binding memory B-cells still detectable seven months post-infection at significantly high levels (Turner et al., 2021). The severity of SARS-CoV-2 has been associated with significantly low levels of total B cells. Another study showed a decrease in neutralizing antibody titers with a significant decline between 6 and 9 months (Kim et al., 2022). Assessment of the risk of reinfection using meta-analysis of observational descriptive cohort studies revealed a protection efficacy of 85%, which increased to 91.7% when considering only symptomatic infection (Petras, 2021). Another essential element of

immunity is understanding the extent of cross-protection against VOC. Serum neutralizing ability from previously infected individuals was shown to be four to sixfold less potent against the Beta and Delta variants than the Alpha variant (Planas et al., 2021). The effectiveness of natural infection in protecting against Omicron variant was 27.5% and 38.3% in individuals infected before the predominance of the Delta variant (Chin et al., 2022).

Several vaccines have been approved for emergency use, including Pfizer-BioNtech (BNT162b2), Moderna (mRNA-1273), Johnson and Johnson (Ad26.COV2.S), AstraZeneca (AZD1222), and more. Multiple studies have shown that vaccines effectively protect against severe disease, hospitalization, and death, with neutralizing antibodies indicating vaccine efficacy (Andrews et al., 2022; Wu et al. 2022; Cona et al., 2023). Neutralizing responses post-BNT16b2 vaccine showed a significant increase in neutralizing antibodies 28 days post-initial dose, with an average half-life of ±68 days. However, immunity was short-lived (Maeda et al., 2021). A six-month longitudinal profile on healthcare workers post-two doses of BNT162b2 vaccination showed significantly higher neutralizing antibodies six weeks post-vaccination, where vaccinated individuals with prior SARS-CoV-2 infection had more potent neutralizing antibodies (Chivu-Economescu et al., 2022). Vaccination was also shown to be 18.6% to 40.9% effective in protecting against Omicron variant (Chin et al., 2022). Assessment of cellular and humoral immune responses in healthcare workers at two weeks and five months post-primary vaccination showed a significant decrease in S1-specific T cell responses, whereas N-specific responses remained comparable between the two collection periods (Faas et al., 2022). Assessment of neutralizing antibodies and anti-receptor binding domain (RBD) IgG also showed a significant decline in responses between two weeks and five months post-primary vaccination (Faas et al., 2022). These studies show that natural and vaccine-induced immunity offers protection against reinfection. However, IgG and neutralizing immunity do appear to decline with time.

The immune response is likely multifactorial, with a role for both the humoral and cellular responses. T-cell responses are crucial in protecting against MERS-CoV and SARS-CoV (Zhao et al., 2017) and will likely prove critical for long-term immune protection against SARS-CoV-2. A study investigating the persistence of memory T-cells found that individuals who have recovered from SARS-CoV still had detectable T-cell responses ten years after infection (Ng et al., 2016). $CD4^+$ T helper cells play a

role in the production of virus-specific antibodies by B cells, and cytotoxic $CD8^+$ T cells function in the killing of host-infected cells (Yang et al., 2004). Studies using BaLB/c mice showed that T-cells play a role in survival and destroying cells infected with MERS-CoV in the lungs, highlighting the importance of T-cells controlling the pathogenesis and outcome of MERS-CoV (Yang et al., 2004). A study on SARS-CoV-2 has associated mild COVID-19 infections with strong T-cell responses (Moderbacher et al., 2020; Sekine et al., 2020). Activation of CD4+ T-helper cells was shown to be comparable in individuals infected with either the Ancestral strain or the Alpha variant (Mazzoni et al., 2022), suggesting that mutations do not affect the entire CD4+ T cell capacity in recognizing the Ancestral S protein. Stimulated peripheral blood mononuclear cells (PBMCs) from individuals previously infected with the Ancestral strain, with peptide pools selectively spanning regions of the Ancestral S protein mutated in the Alpha, Beta, or Delta variant, showed that frequencies of CD4+ T cells specific for VOC reference pools were significantly lower than those specific for the entire Ancestral spike protein (Mazzoni et al., 2022). Similarly, assessment of cellular immunity induced by BNT162b2 and Ad26.COV2.S vaccines, assessed by pooled peptide IFN-γ enzyme-linked immunospot (ELISpot), show substantial cross-reactivity to the Omicron variant similar to the Ancestral strain and Delta variant (Liu et al., 2022). A good clinical outcome and an adequate viral clearance post-SARS-CoV-2 infection is associated with early development of cytotoxic CD8+ T cell responses and high expression levels of effector molecules (Liu et al., 2022).

2.5. Transmission and Epidemiology

CoVs are of zoonotic origin and are suggested to have originated from bats (Li et al., 2005). The viruses are capable of crossing species barriers, causing infection in humans ranging from self-limiting infections to severe disease (Woolhouse et al., 2012). MERS-CoV and SARS-CoV emerged in bats and infected humans through intermediate hosts, including the Himalayan palm civet in SARS-CoV and the dromedary camel in MERS-CoV (Shi and Hu, 2008; Memish et al., 2014). Two hypothesis have been suggested regarding the origin of SARS-CoV-2. The virus could have either originated through zoonosis or a laboratory source, with scientific evidence supports a direct zoonotic origin from bats (Zhou et al., 2020). Additionally, the index cases of SARS-CoV-2 is linked to an animal market, suggesting that SARS-CoV-2 is also of zoonotic origin (Ren et al., 2020). Viruses more closely related to SARS-Cov-

2 have also been isolated in bats and pangolins in China, Thailand, Japan, and Cambodia (Wacharapluesadee et al., 2021; Hul et al., 2021). Thus, animal-to-human has been identified as the main transmission mechanism of CoVs in human populations. Subsequent cases of these viruses have been associated with close contact between humans. Transmission between humans is through respiratory droplets from coughing or sneezing. Infections can also be through contact with infected fomites (surfaces). Figure 3 illustrates the transmission cycle of CoVs. Table 1 give a comparative overview of three different viruses and how they compare to influenza virus which occurs annually.

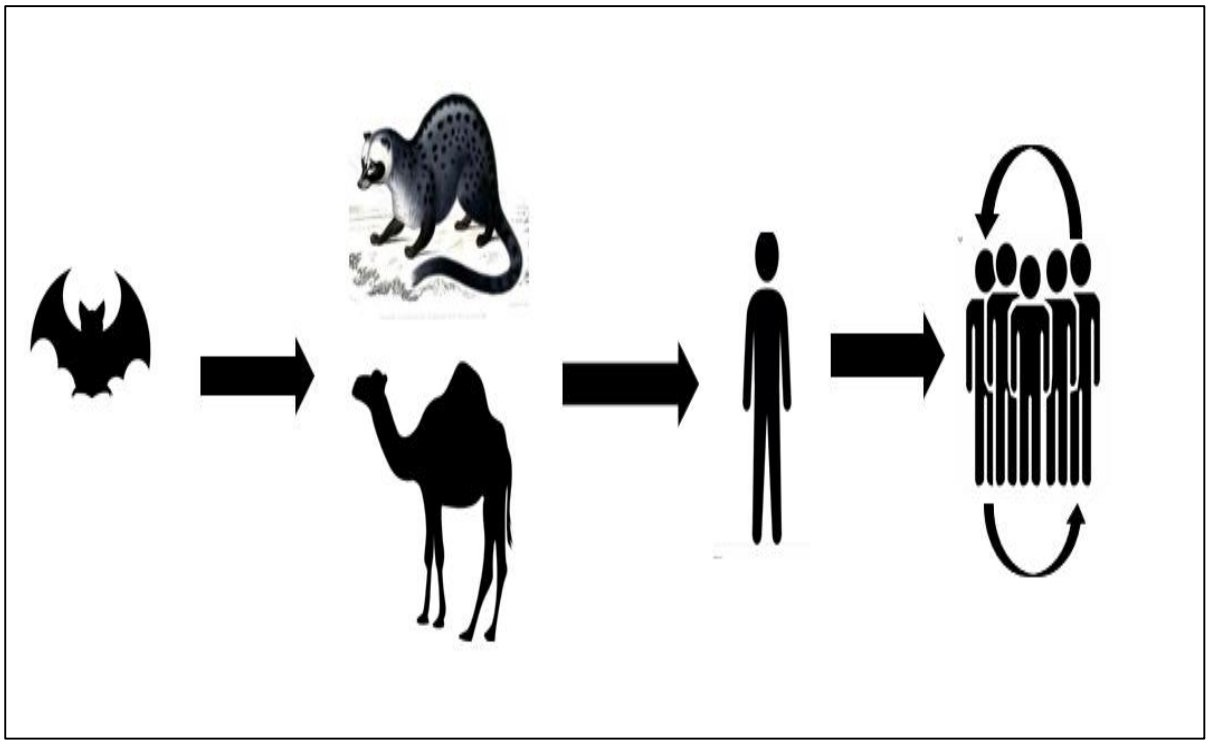

Figure 3: Transmission cycle of coronaviruses (CoVs) to humans. CoVs are of zoonotic origin and are capable of crossing species barriers. The viruses emerged in bats and infected humans through an intermediate. Once introduced into the human population, the viruses are transmitted between humans via respiratory droplets and contaminated fomites (Shi and Hu, 2008; Memish et al., 2014)

Table 1: Comparative Overview of SARS-CoV-2, MERS-CoV, SARS-CoV, and Influenza Virus

Clinical Characteristic	SARS-CoV-2	MERS-CoV	SARS-CoV	Influenza Virus
Emergence	December 2019	June 2012	November 2022	Various strains, seasonal
First detection	Wuhan, China	Jeddah, Saudi Arabia	Guangdong, China	
Cases	~774 million	~2000	±8000	±1 billion
Deaths	~7 million	858	±700	290,000 to 650,000 annually
Fatality rate	<0.1%	37%	9%	Typically <0.1%
R_0 (Basic Reproduction number)	Estimated 2.2	Estimated between 0.3 and 0.8	2 during the outbreak	1.3-1.8 (seasonal)
Gobal impact	Widespread pandemic	Limited outbreak	Limited to specific regions	Seasonal outbreaks

2.5.1. Severe Acute Respiratory Syndrome Coronavirus

SARS-CoV emerged in Guangdong, China, in mid-November 2002 (Fouchier et al., 2003). The index cases of the outbreak involved food handlers and chefs working in restaurants where exotic animals were slaughtered. Late February 2003, cases of the virus were reported in Hong Kong and began to spread to other countries (Lee et al., 2003). Cases of pneumonia were reported in Hong Kong, Canada, Vietnam, and Singapore between February and March 2003, all linked to single index patient staying at a hotel in Hong Kong between 21 and 22 February 2003 (Wong and Hui, 2004; Vu et al., 2004). During the outbreak, the virus spread rapidly by air travel to 29 countries, with over 8000 cases and more than 700 fatalities. The zoonotic origin of the virus was traced back to a wet animal market where viruses closely related to SARS-CoV were detected in small mammals, including Himalayan palm civets, raccoon dogs, and other mammals (Tu et al., 2004; Song et al., 2005; Shi and Hu, 2008). Additionally, individuals working in these markets had a high level of antibodies to SARS-CoV. Further work was established to catch wild civets. No SARS-CoV infection was found within these animals, suggesting they were intermediate hosts (Shi and Hu, 2008). SARS-CoV and other related CoVs have been identified in bats, suggesting that they are the true reservoirs of these viruses (Li et al., 2005).

2.5.2. Middle East Respiratory Coronavirus

MERS-CoV was first detected in Saudi Arabia in 2012. The virus was initially known as Human coronavirus- Erasmus Medical Centre (HCoV-EMC) and isolated from a patient presenting with fever, shortness of breath, cough, and expectoration who later died from respiratory failure in June 2012 (Osterhaus et al., 2012). The following patient from Qatar, a 49-year-old patient, was diagnosed in September 2012 (Danielsson, 2012). The virus from both the patients shared 99.5% identity and was designated MERS-CoV (Danielsson, 2012). A retrospective investigation of samples from hospitalized patients who suffered from severe respiratory illness in April 2012 in Jordan was confirmed as MERS-CoV positive cases (Al-Abdallat et al., 2014). In April 2014, 515 cases MERS-CoV cases were reported in Saudi Arabia (WHO, 2014). A total of 113 cases were also reported in 2014. Studies into the outbreaks suggested that MERS-CoV followed a seasonal distribution, cases were reported in April 2012 in Jordan, between April and May 2013 in Al-Hasa, and the between April and May 2014 in Jeddah (Memish et al., 2013; Rabeeah et al., 2013; Swerdlow et al., 2015). Most MERS-CoV outbreaks have been reported due to secondary household and hospital contact (Drosten et al., 2014). Outbreaks of MERS-CoV have been reported in South Korea, Saudi Arabia, and the United Arab Emirates (Rabeeah et al., 2013; Cowling et al., 2015). Studies tracing the zoonotic origin of MERS-CoV identified camels as a possible source of MERS-CoV (Memish et al., 2014). Serological studies showed high levels of MERS-CoV antibodies in dromedary camels in multiple countries, including Saudi Arabia, Egypt, Qatar, the United Emirates, Nigeria, and Ethiopia (Alagail et al., 2014; Hemida et al., 2014; Reusken et al., 2014). Studies in dromedary camels in Saudi Arabia suggested that pre-existing neutralizing antibodies do not protect against infection (Hemida et al., 2014). These studies presented a possible transmission between camels and humans, but the transmission route has not yet been identified (Azhar et al., 2014; Memish et al., 2014).

2.5.3. Severe Acute Respiratory Syndrome Coronavirus 2

SARS-CoV-2 emerged in Wuhan, China, at the end of 2019 (Bassetti et al., 2020). Between mid-December and December 29, 2019, patients with acute respiratory distress were hospitalized, presenting with fever and cough. The cases were linked to a wet animal market (Ren et al., 2020). At the beginning of January 2020, 41 patients

were confirmed positive for SARS-CoV-2 (Huang et al., 2020; Lu and Stratton, 2020). Towards the end of January, 7000 cases of COVID-19 were reported in China, with 56 fatalities (Bassetti et al., 2020). Genome analysis of the SARS-CoV-2 shows 88% identity to bat-derived SARS-like CoV (Lu et al., 2020). Respiratory droplets are the main route of transmission. The virus can also be transmitted through aerosol droplets and contact with contaminated fomites (Geng and Wang, 2023). The reproduction number (R0) is approximately 2.2, as determined from early case tracking at the onset of the pandemic (Harrison et al., 2020). The doubling time for cases was found to be five days. Cases are still increasing worldwide, with over 770 million infections and over 6.9 million fatalities recorded (https://www.worldometers.info/coronavirus/). Although the pandemic has been declared over, new cases continue to be confirmed worldwide. Most cases are reported in Europe, followed by the West Pacific, Americas, South-East Asia, Eastern Mediterranean, and Africa. Figure 4 is a schematic representation of the accumulative cases per region at the end of July 2023, according to the WHO. SARS-CoV-2 has spread to 217 countries and territories worldwide, with case fatality rates of about 0.90%. South Africa has the highest reported cases in Africa, a cumulative total of 4 million as of July 30, 2023, with 102 595 fatalities (http://www.gov.za/). This may be a reflection of the availability to perform diagnostic testing and reporting, which was considered a priority and this availability may have been lacking in lower resource countries. The epicenter of the outbreak in South Africa was the Gauteng province, with 1 356 959 cumulative cases to date. Overall, there is a 97% recovery rate reported in South Africa. Figure 5 shows the total number of cases in South Africa, including the recoveries and fatalities as of February 2023 (http://www.nicd.ac.za/disease-a-z-index/covid-19/). The virus continues to circulate however, the rate of testing is significantly reduced, and the actual number of cases could be higher than currently recorded as the virus circulates with other seasonal respiratory viruses.

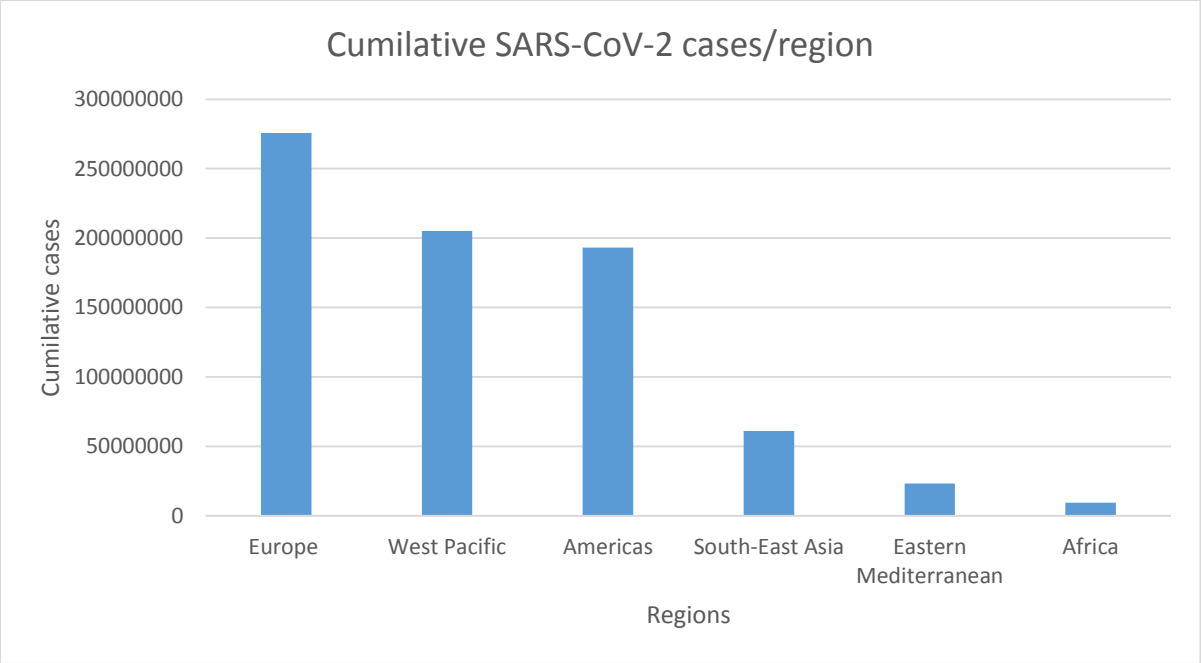

Figure 4: SARS-CoV-2 cumulative cases for each region as of July 2023. *WHO COVID-19 Dashboard. Geneva: World Health Organization, 2020. Available online: https://covid19.who.int/.*

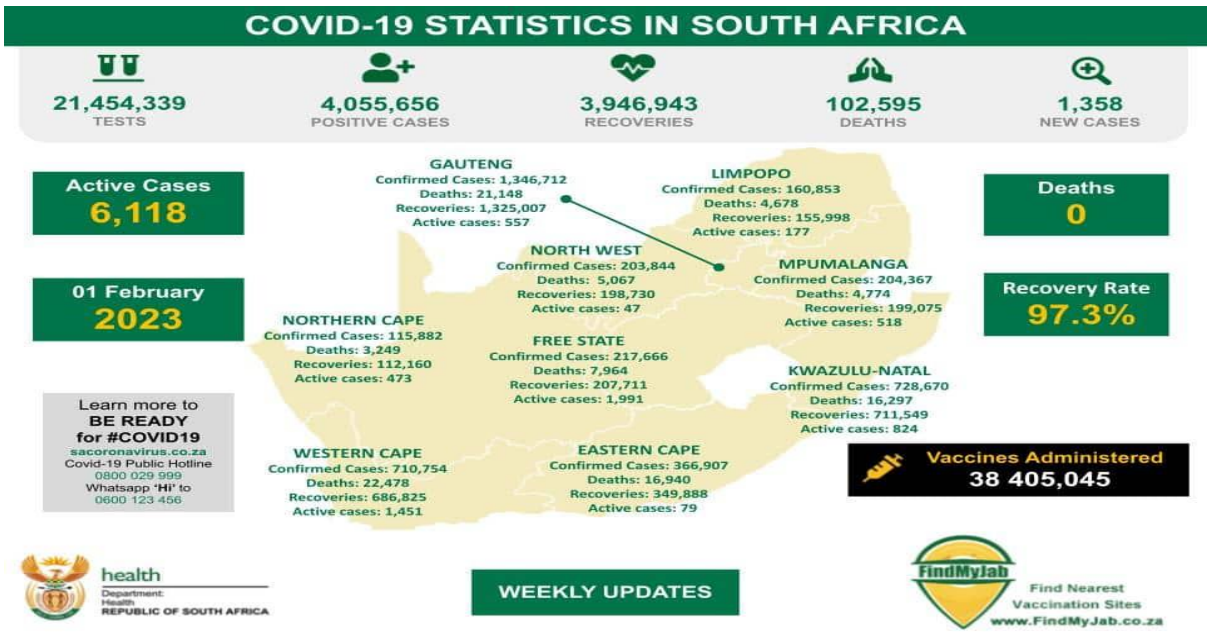

Figure 5: Cumulative SARS-CoV-2 cases in South Africa as of February 2023 (http://www.nicd.ac.za/disease-a-z-index/covid-19/).

2.5.3.1. Variants of Concern

SARS-CoV-2 has an RNA proofreading mechanism during replication, which previously was thought would provide relative protection from the rapid development of viral mutations (Mack et al., 2023). Despite this, a wide range of mutations have been observed, and the WHO has identified five lineages as VOC globally. These variants carry different mutations within the S protein and the RBD, resulting in increased transmissibility, virulence, or immune resistance. The Alpha (B.1.1.7) variant, first described in the United Kingdom, has 23 mutations in the spike protein. Although the impact of all the changes is not known, RBD N501Y appears to increase the affinity of binding ACE-2 receptor, making the virus more infectious (Davies et al., 2021). The Beta lineage, first identified in South Africa, has eight mutations in the S protein, with three substitutions in the RBD (E484K, K417N, and N501Y), resulting in increased transmissibility and immune escape(Tegally et al., 2021). Initially identified in India, the Delta variant has critical mutations, including D614G, L452R, P681R, and T478K in the S protein, resulting in enhanced transmissibility and immunity to neutralizing antibodies (Dhawan et al., 2022). The Omicron variant (B.1.1.529), first detected in November 2021 in Botswana, carries at least 32 mutations on the S protein, with 15 occurring within RBD (Cao et al., 2022).

2.6. Clinical manifestation and laboratory diagnosis

Accurate diagnosis of CoV is essential to ensure appropriate patient management, to control local transmission, and as a public health measure to reduce the global spread of viruses during an outbreak. Current data indicate that older adults and immunocompromised individuals are at greater risk for severe COVID-19 illness than younger individuals (Mardani et al., 2023). The majority of critical patients have pre-existing comorbidities, including diabetes, chronic respiratory infection, and cardiovascular diseases (Huang et al., 2020). The incubation period of CoVs is long, ranging from 2-5 days for COVID-19, before symptom onset, during which the virus can spread. While some cases are asymptomatic, most individuals present with mild to moderate infection characterized by respiratory symptoms. Early clinical signs include fever, myalgia, headache, sore throat, malaise, and coughing, which can progress to shortness of breath (Wang et al., 2020). These symptoms can progress to more severe diseases, including pneumonia, septic shock, sepsis, and acute respiratory failure (Wang et al., 2020). Severe disease usually begins one week post-

symptom onset. The primary manifestation is shortness of breath caused by hypoxemia, which eventually leads to respiratory failure and meets the criteria for ARDS (Wang et al., 2020). These patients exhibit elevated levels of proinflammatory cytokines, including IL-1, IL-6, IL-8, and TNF-α, and elevated concentrations of inflammatory markers, including D-dimer, ferritin, and C-reactive protein (CRP) (Sakthivadivel et al., 2021). Severe disease can lead to extrapulmonary disease, including acute cardiac, kidney, and liver injury, cardiac arrhythmias, shock, or death (Babapoor-farrokhran et al., 2020). In patients who survive, the recovery time varies depending on disease severity and other host factors, including age and pre-existing health conditions (Kassie et al., 2023). In individuals with mild to moderate disease, recovery is usually two to three weeks after symptom onset. In patients with severe disease, recovery could take several weeks to months. In contrast, some may experience lingering symptoms known as long COVID or PASC of SARS-CoV-2 infection. These long-term symptoms can persist for weeks, months, or years after the initial infection, causing fatigue, shortness of breath, and muscle weakness (Bowe et al., 2023).

The first step in managing an infection is rapid, sensitive, and accurate detection of SARS-CoV-2. There are different techniques available for clinical diagnosis of viruses. The primary phase of infection corresponds to the viremic period of the disease. This allows detection by either virus isolation or detection of the viral nucleic acid using molecular techniques. Viral nucleic acid detection methods such as real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) target a specific viral genomic region, making it a highly sensitive method with rapid turnaround time. qRT-PCR is considered the gold standard for COVID-19 diagnosis (Oliveira et al., 2023). Various qRT-PCR kits have been developed since the beginning of the COVID-19 pandemic, varying in test sensitivity and specificity, targeting either the S, N, or both proteins using respiratory secretions or nasopharyngeal aspirates. However, due to the constant mutations of SARS-CoV-2, results from qRT–PCR can sometimes be affected by viral RNA sequence variation, yielding false-negative results. Studies on SARS-CoV-2 indicate that viral loads peak in the second week of infection. Because the viral load may be low during sampling, it is essential to use sensitive techniques that would be able to detect viral nucleic acid even at very low levels. Virus isolation is the most reliable method for diagnosis. However, it does not have a diagnostic

application as it requires biosafety (BSL) 3 facilities. Virus isolation can be done *in vitro* using a susceptible cell line such as African green monkey kidney cells (Vero). CoVs can be cultured in various continuous cell lines, including Vero cells, resulting in cytopathic effects (CPE). Rapid diagnostic assays for the detection of SARS-CoV-2 antigen have been developed (Mak et al., 2020; Porte et al., 2020). The antigen present in the sample binds to antibodies affixed to a paper strip enclosed in a cassette. This reaction generates a visually detectable signal in less than an hour. The antigen is detected if the virus is actively replicating. Thus, the tests can be used to identify acute or early infection.

Post the viremic phase, infection is confirmed by serological techniques, including immunofluorescence assay (IFA), lateral flow immunoassay (LFIA), chemiluminescence immunoassay (CLIA), and enzyme-linked immunosorbent assay (ELISA). Serological testing complements virus detection, indicating past infection by detecting IgM or IgG antibodies. Detection occurs through an enzyme reaction or fluorescein-conjugated to a secondary antibody in ELISA and IFA. Because antibodies can be detected for a more extended period of time, the development of serological assays for the detection of SARS-CoV-2 is essential. Serology not only plays a role in diagnosis, but it also plays a pivotal role in managing the SARS-CoV-2 pandemic through contact tracing, vaccine development, epidemiological studies, and assessing herd immunity, guiding public health policies (Goudsmit, 2020; Theel et al., 2020, Horbach et al., 2024). Various serological assays for detecting SARS-CoV-2 antibodies were developed and validated at the beginning of the pandemic (Muench et al., 2020; Kang et al., 2021). The validation of assays is crucial for emerging viruses, especially during outbreaks where rapid development of assays is required. Validating an in-house assay ensures accurate and reliable results, as the validation process ensures that the assay consistently measures what it is intended to measure. Additionally, developing an in-house assay allows a more direct quality control by optimizing each step of the assay, ensuring accuracy, reproducibility, and reliability of results. It is also more cost-effective, especially for long-term research projects, as commercially available assays can be expensive.

The development of ELISA using recombinant antigen for the detection of IgM and IgG have been developed targeting either the S or the N protein (Sapkal et al., 2020; Sil et al., 2021). IgM levels increase during the first week after SARS-CoV-2 infection,

peak after two weeks, and then fall back to near-background levels in most individuals (Zhao et al., 2020). Therefore, IgM antibodies are a useful marker for recent infection and advantageous as the antibodies are only detectable in a recently infected patient. IgG is detectable after one week and maintained at a high level for longer. Acute infection can be confirmed by a fourfold increase in antibody titer between two paired samples taken two weeks apart. The use of neutralizing antibody tests indicates that high levels of neutralizing antibodies are found against SARS-CoV-2 and persist for several months post-infection (Wajnberg et al., 2020), but antibody titers decline with time (Vanshylla et al., 2021).

The plaque reduction neutralization test (PRNT) is the gold standard for neutralizing antibody detection (Horbach et al., 2024). PRNT is an *in vitro* assay that can quantify type-specific neutralizing antibodies against a virus. This is done by mixing a constant volume of a virus with dilutions of serum samples or a solution of antibodies. A susceptible cell line, such as Vero cells for SARS-CoV-2, is then inoculated with a mixture of virus and antibodies. The concentration of plaque-forming units (PFU) can be determined based on the number of plaques that form during incubation. PFU can be measured by fluorescent antibodies, microscopic observation, or specific dyes that react with the infected cells (Horbach et al., 2024; Souza et al., 2021). However, neutralization assays for SARS-CoV-2 require a high containment laboratory such as a BSL 3 due to the highly infectious and pathogenic virus. The Pseudovirus neutralization assay system is a useful alternative approach to screening for neutralizing antibodies within a BSL 2 laboratory (Wohlgemuth et al., 2021). Pseudovirus refers to a retrovirus that can integrate the glycoprotein of another virus to form a virus with an exogenous virus envelope with the genome retaining the characteristics of the retrovirus. Upon entry into a susceptible host cell, pseudoviruses can only replicate once and lack the virulent components of the wild-type virus. These properties allow for the use of pseudoviruses in the BSL-2 laboratory. Several packaging systems have been used for the generation of SARS-CoV-2 pseudoviruses. These include the lentiviral vector system, which originates from the human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), and vesicular stomatitis virus (VSV) (Hu et al., 2020; Nie et al., 2020; Donofrio et al., 2021).

Parallel testing of samples of other infections capable of causing similar clinical picture, including influenza and *Mycoplasma pneumonia,* needs to be performed for

differential diagnosis in the early stages of the disease (Chaudhry et al., 2021). The major disadvantage in accurate diagnosis is the long incubation period of CoV before the onset of symptoms and other individuals being asymptomatic.

2.7. Treatment and control

In an attempt to control the pandemic, vaccines designed using the viral sequence of the Ancestral (Wuhan) strain were developed. These vaccines were developed using either pre-existing or novel technologies. The primary vaccines used worldwide include those developed by Pfizer, Moderna, Johnson and Johnson (J&J), Novavax, Oxford/AstraZenica, and Spikevax. Pfizer and Moderna developed mRNA vaccines, while J&J used an adenovirus vector. The Novavax (NVX-CoV2373) vaccine is a protein adjuvant that can be administered to individuals 12 years and older. The Spikevax vaccine is an mRNA bivalent (original/Omicron) developed by Moderna. Current data has shown that these vaccines protect against severe disease, reinfection, and death.

2.7.1. BNT162b2 by Pfizer-BioNTech

The Pfizer vaccine contains a modified nucleoside mRNA that encodes the SARS-CoV-2 S protein, delivered in a lipid nanoparticle. The modified nucleosides function to avoid early activation of interferon-associated genes. Phase I/II clinical trials took place in May 2020 and involved 45 participants grouped into three groups of 15 participants each. The study determined the immunogenicity of the vaccine where group 1 was administered two 10µg doses 21 days apart. Group 2 had two 30µg doses, separated by 21 days, and group 3 had a single 100µg dose (Mulligan et al., 2020). Additionally, a group of recovered individuals had their IgG antibodies quantified. Participants were tested for RBD IgG antibodies 35 days after administration of the first dose. Group 1 demonstrated an RBD IgG concentration of 5880U/ml, group 2 16166U/mL, and group 3 1260U/mL compared to the recovered group that demonstrated a RBD IgG concentration of 602U/mL. The trial showed that the Pfizer vaccine effectively produced antibodies against SARS-CoV-2 (Mulligan et al., 2020). This was followed by a phase III clinical trial involving 43448 participants 16 years or older, of which 21720 received two 30µg doses of the BNT162b2 vaccine separated by 21 days and 21728 received placebos. Phase III trial was concluded in

November 2020, and the vaccine showed 95% efficacy (Polack et al., 2020). On December 11 2020, the USA FDA approved the emergency use of the vaccine.

2.7.2. mRNA-1273 by Moderna

Moderna vaccine also uses mRNA technology with a similar mechanism of action to Pfizer. Phase I of the clinical trial for the vaccine began on March 16 2020 and, on May 12 2020, received the FDA fast-track designation for mRNA-1273. The phase II clinical trial involved 600 healthy individuals grouped into two cohorts of 300 (≥18 to <55 and ≥55). The groups were assigned in a 1:1:1 ratio to either receive 50µg, vaccine, or placebo, administered 28 days apart. The administration of mRNA-1273 in two doses (50mg and 100mg) elicited substantial immune responses against SARS-CoV-2, confirming the safety and immunogenicity of the vaccine (Chu et al., 2020). Phase III began on July 26 2020. On May 10 2021, the FDA approved Moderna's emergency use in adolescents between 12 and 18 years old. A total of 30420 participants were recruited for the study and assigned at a 1:1 ratio to receive a 100 µg vaccine or the placebo, 28 days apart. The studies showed that the vaccine was 94.1% effective in protecting against disease (Sapkal et al., 2020). However, efficacy in the elderly, >65 years, was slightly lower at 86.4%. Evaluation of serum neutralizing activity against B.1.1.7 (Alpha variant) and B.1.351 (Beta variant) showed that the vaccine maintains its neutralizing activity against the B.1.1.7 variant, but there is a decreased titer against the B.1.351 variant.

2.7.3. ChAdOx1 nCoV by Oxford/AstraZeneca

The ChAdOx1 nCoV-19 vaccine (AZD1222), developed at Oxford University, consists of a replication-deficient chimpanzee adenoviral vector ChAdOx1 (Dicks et al., 2012), containing the SARS-CoV-2 S glycoprotein gene. Phase 1/2 of the clinical trial was carried out between April 23 and May 212020, in the United Kingdom (UK). The trail was a single-blind, randomised controlled trial, where participants (n=1077) received either a single intramuscular injection of the ChAdOx1 nCoV-19 vaccine (n=534) or MenACWY vaccine (n=534). Ten participants were also enrolled in a non-randomised ChAdOx1 nCoV-19 prime-boost group. Phase 1 of the clinical trial investigated the safety and immunogenicity of the vaccine and follow up were on t days 3, 7, 14, 28, and 56 after vaccination. ChAdOx1 nCoV-19 vaccination resulted in no adverse events. Neutralizing antibodies were detected in 91% of individuals after a single dose

and in all individuals after booster vaccination (Folegatti et al., 2020). The clinical trial was expanded to South Africa (SA) and Brazil (Voysey et al., 2021). This included 23848 participants, randomly assigned 1:1 to receive either ChAdOx1 nCoV-19 vaccination or MenACWY control vaccine. Participants receiving the ChAdOx1, received two doses of $5x10^{10}$ viral particles which showed vaccine efficacy of 62.1% (Voysey et al., 2021). A subset of participants, in the UK, received half dose of the first vaccine and standard for the second, resulting in vaccine efficacy of 90% (Voysey et al., 2021). The vaccines is primarily distributed in India, Brazil, and the UK.

2.7.4. Ad26.COV2.S Johnson & Johnson SARS-CoV-2 Vaccine

The J&J vaccine was designed with the adenovirus vector technology previously used for the development of an Ebola vaccine in West Africa (Mendonça et al., 2021). In order to accelerate the vaccine development process, phase I/II was combined and began in July 2020 to assess the safety and dosage of the vaccine with participants in the USA and Belgium. Phase III began on September 27, 2020, with 44325 participants from SA, Brazil, and the US. The group was divided into 19630 individuals receiving the Ad26.COV2S and 19631 receiving the placebo. Results showed that the vaccine protected against moderate to severe disease when infection occurred at least 14 days post-vaccine administration (Sadoff et al., 2021). After preliminary Phase III results were released, the FDA approved the emergency use of the J&J vaccine in the US. As of May 2021, the J&J vaccine is being manufactured widely and used worldwide. SA began the administration of the vaccine on February 17, 2021. The vaccine was approved earlier in South Africa due to the emergence of the variant 501.V2 (Beta variant).

2.7.5. Antiviral Therapy

Developing effective antiviral therapy is essential, especially for immunocompromised individuals who do not respond well to vaccination (Shoham et al., 2023). Since the onset of the pandemic, extensive research has been conducted to find effective treatments for curbing the spread of COVID-19. Several antivirals have been explored and approved for emergency since the beginning of SARS-CoV-2. Some of these include remdesivir, monoclonal antibodies, convalescent plasma, and traditional medicines. A significant portion of this research has focused on repurposing existing antiviral drugs developed for infections like HIV, including HIV-1 protease inhibitors

like lopinavir, hepatitis C virus protease inhibitors such as danoprevir, influenza antiviral favipiravir, and the RNA polymerase inhibitor remdesivir. As part of these efforts, traditional medicines have emerged as promising, cost-effective alternatives for patients afflicted with COVID-19, although the efficacy of traditional medicines is not known. In the early stages of the pandemic, various countries explored using plant extract-based traditional medicines as a treatment. These remedies were considered affordable and accessible options in the fight against the novel coronavirus.

Remdesivir has broad-spectrum activity against multiple RNA viruses, including SARS-CoV, MERS-CoV, and SARS-CoV-2 (Beigel et al., 2020; Sheahan et al., 2020). The drug was initially designed for the treatment of Ebola virus (Warren et al., 2016). During the early stages of the COVID-19 pandemic, it was one of the first drugs to show promise against SARS-CoV-2 in laboratory studies (Beigel et al., 2020). Subsequently, numerous clinical trials were conducted to evaluate its effectiveness in treating COVID-19 patients. A study found that remdesivir had a modest but significant effect in reducing the recovery time of hospitalized COVID-19 patients compared to a placebo (Beigel et al., 2020). A meta-analysis of mortality on remdesivir clinical trials showed that the drug had no significant effect on patients with COVID-19 already ventilated, but daily remdesivir infusions in other hospitalized patients could somewhat reduce the risk of death (WHO Solidarity Trial Consortium, 2022).

Neutralizing monoclonal antibodies are recombinant proteins derived from B cells of convalescent patients or humanized mice that can treat COVID-19. Monoclonal antibodies like casirivimab/imdevimab and bamlanivimab/etesevimab have been developed to target the S protein of SARS-CoV-2 (Falcone et al., 2021). These antibodies have been used under European University Association (EUA) to treat mild to moderate COVID-19 cases, especially in patients at high risk of progression to severe disease. REGN-COV2, a combination of casirivimab and imdevimab, was shown to reduce viral load in patients with high viral load or whose immune response had not yet been initiated (Weinreich et al., 2021). The use of bamlanivimab/etesevimab was shown to reduce the risk of hospitalization and death in high-risk patients and accelerate the decline in viral load (Dougan et al., 2021).

Immune modulators are drugs that alter an immune response by either enhancing or suppressing immunity. Immune modulators are synthetic or biological substances that can modulate components of the immune system, both the innate and the adaptive immune system. The nature of immune modulators is that the modulating substance results in an immunomodulating effect when used at different concentrations and times of infection. Several medicinal plants have also been used to treat and improve various infections. Phela is a traditional herbal medicinal plant under development as an immune booster in South Africa. Phela is prepared from four South African traditional medicinal plants by combining *Gladiolus dalenii*, *Senna occidentalis, Rotheca myriciodes,* and *Clerodendrum glabrum* at specific ratios. Phela is produced by milling to a homogeneous consistency, then sterilized by irradiation and provided as a powder in a capsule at a final concentration of 350mg/capsule. In an animal study, Phela was shown to stimulate the immune system in immune-suppressed rodents, illustrating that it can act as an immune stimulant to a compromised immune system (Lekhooa et al., 2012). In these rodents, Phela restored cyclosporine-induced immunosuppression, indicating activation of IL-2. The leaves of *Clausena harmandiana* were shown to inhibit CoV entry by acting directly on the viral particle, making it a possible new source of antiviral agents against human CoVs (Chambon et al., 2023). Artemisinin, an antimalarial drug derived from a Chinese herb, was shown to reduce the production of SARS-CoV-2 protein and block SARS-CoV-2 infection at the post-entry level (Cao et al., 2020). Antiviral studies on traditional medicines from Ayurveda found seven active compounds that could inhibit the activity of the S glycoprotein (Maurya et al., 2022), suggesting these drugs could be used as potential therapy for COVID-19. The effectiveness of these drugs shows promise and further safety studies are warranted.

2.8. Conclusion

The immune correlates of protection against SARS-CoV-2 are still not yet fully understood. Although the pandemic has been declared over, SARS-COV-2 is still circulating, and understanding the immune correlates of protection is an important factor for understanding the disease and developing vaccines and antiviral therapy. Longitudinal studies tracking antibody and T-cell immune responses in recovered individuals over time will be essential in providing insight into the duration and effectiveness of immunity in protecting against SARS-CoV-2. Additionally, as

individuals respond differently to infection, it is also necessary to investigate and understand factors influencing varying immune responses among individuals. Thus, further understanding of SARS-CoV-2 immunity is essential for optimizing current vaccination strategies, including booster doses, and designing future vaccines and therapeutics for CoV-related outbreaks. Other emerging or re-emerging pathogens have the potential to cause pandemics, such as the SARS-CoV-2. Emerging pathogens of zoonotic origin, such as SARS-COV-2, require a multifaced approach involving surveillance, research, and pandemic preparedness. Enhancing global surveillance of emerging viruses is critical to swiftly detecting, monitoring, and responding to potential pandemics. This can be done by monitoring wildlife and domestic animals for signs of emerging viruses and by implementing widespread genomic sequencing to track the evolution of viruses, which would help in tracking the origin and transmission patterns. Although serological tests may not be ideal for diagnosis of SARS-CoV-2, they are an essential tool for surveillance. As the virus continues to evolve and circulate, detection of antibodies will be essential to track population immunity against natural and vaccine-acquired immunity, which will play a role in policy making regarding booster vaccination. There are different serological platforms available, including ELISA, IFA, and LFA that are commercially available, for detection of IgG antibodies for surveillance. However, because the virus continues to mutate, validating the assays for recent variants may be important as the sensitivity may decrease. The S protein is responsible for binding of virus to the host and the main target for developed vaccines. Surveillance of hybrid immunity and vaccine-induced immunity can be done by targeting the S protein. The N protein plays an essential role in pathogenesis and virus replication and can be used for surveillance of natural infection immunity. Both proteins are highly immunogenic and will play an essential role in surveillance for SARS-CoV-2. Finally, by promoting standardized reporting protocols, sharing data transparently, and investing in research and development, the scientific world can proactively identify emerging viruses, monitor immunity, enable timely interventions to pandemics, and safeguard public health worldwide.

Authors Contribution

M Litabe: Writing- original draft, reviewing and editing

Burt FJ: Writing– review and editing

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Chapter 3
Development and validation of SARS-CoV-2 anti-spike IgG in-house assays

Matefo Litabe[a], Cloete van Vuuren[bc], Philip Armand Bester[ad], Dominique Goedhals[de], Samantha Potgieter[b], John Frater [fh], Craig Thompson[gh], Daniel Wright[i j], Theresa Lambe[ij], Sunetra Gupta[gh], Maréza Brink[b], Danelle van Jaarsveldt[a], Felicity Jane Burt[ad]

a. Pathogen Research Laboratory, Division of Virology, Faculty of Health Sciences, University of the Free State, Bloemfontein, South Africa
b. Department of Internal Medicine, Faculty of Health Sciences, University of the Free State, Bloemfontein, South Africa
c. 3 Military Hospital, Bloemfontein, South Africa
d. Division of Virology, National Health Laboratory Service, Bloemfontein, South Africa
e. PathCare Vermaak, Pretoria, South Africa
f. Oxford NIHR Biomedical Research Centre, Oxford, United Kingdom
g. Department of Zoology, University of Oxford, Oxford, United Kingdom
h. Peter Medawar Building for Pathogen Research, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom
i. Chinese Academy of Medical Science (CAMS) Oxford Institute, University of Oxford, Oxford, United Kingdom
j. The Jenner Institute, University of Oxford, Oxford, UK, University of Oxford, Oxford, United Kingdom

Abstract

Using serological assays to detect antibodies plays a vital role in surveillance during an outbreak. Effectively determining SARS-CoV-2 seroprevalence within a population is important as it helps to improve our understanding of the dynamics of virus circulation, to identify individuals at risk of infection, and the extent of virus exposure in the community. Commercial assays are expensive and hence in-house assays could have a role in low resource countries. The study aimed to develop and validate two anti-spike in-house assays, enzyme linked immunosorbent assays (ELISA) and immunofluorescence antibody (IFA). A total of 89 samples were collected from COVID-19 PCR-confirmed patients between days 2–94 after onset of symptoms. A

100 pre-pandemic samples were used as a negative control panel to determine a suitable cutoff to differentiate negative from positive samples. The assays demonstrated 96% and 100% specificity for ELISA and IFA, respectively. The assays exhibited a sensitivity of 100% for ELISA and 98.8% for IFA when testing samples collected more than one week after the onset of symptoms. The positive predictive values were 92.1% for ELISA and 91.0% for IFA on PCR-confirmed positive samples. A further 62 samples were collected from different COVID-19 waves between November 2020 and October 2021. The samples were used to determine if the assay could detect IgG antibodies against different circulating SARS-CoV-2 variants. All samples tested positive, regardless of the infecting variant. High concordance was demonstrated between the laboratory-developed assays and the commercial immunoassay using samples collected from South African populations. Results suggest that both the in-house ELISA and IFA can be used for surveillance and testing of SARS-CoV-2 IgG antibodies, especially for the analysis of serum samples of patients taken one week post-onset of symptoms.

1. Introduction

In December 2019, Severe Acute Respiratory coronavirus 2 (SARS-CoV-2), a novel coronavirus (CoV) was first detected in Wuhan, China. The virus is a positive sense ribonucleic acid (RNA) virus with four main structural proteins, namely nucleocapsid (N), Spike (S), Envelope (E), and Membrane (M) protein. The N and S proteins are highly immunogenic and result in the production of immunoglobulin (Ig) M and IgG antibodies (Li and Li, 2021). Thus, these proteins are used for the development of serological assays. Determining the level of immunity after SARS-CoV-2 infection or post-vaccination remains crucial. The use of serological assays for detecting antibodies against viral infection plays a vital role in surveillance during and after an outbreak. Serological assays assist in effectively determining SARS-CoV-2 seroprevalence within a population, which helps improve understanding of the dynamics of virus circulation, identify individuals at risk of infection, and also help identify the extent of virus exposure within a community, which could help guide control measures. Serological assays can also assist in determining the possible level of immunity following infection. The main target proteins for the development of serological assays against SARS-COV-2 include the N, the full-length S glycoprotein, and the receptor-binding domain (RBD) of the S protein. The timing for seroconversion

is vital in determining the optimal time for sample collection. It is essential to know when different antibodies against SARS-CoV-2 become detectable (seroconversion), how their concentrations change over time, and how long they last. Studies show that IgA antibodies are detectable between 3 and 6 days post-symptom onset (Sterlin et al., 2021). Seroconversion is observed between 10 and 13 days for IgM and 12 and 14 days for IgG antibodies (Zhao et al., 2020).

Serology provides essential information for understanding critical aspects of SARS-CoV-2 infection. Measuring the level of protection conferred by SARS-CoV-2 total Ig and IgG antibodies has been reported to be reliable. The aim of the study was to develop and validate an in-house IgG IFA and ELISA against the SARS-CoV-2 S protein to identify potential correlates of immunity post-exposure to SARS-CoV-2 in the South African population. It is essential to validate an assay using serum samples from the country where it will be used, especially in African countries, as there is less reporting on serological assays (Ndaye et al., 2021). The SARS-CoV-2 S protein used for the study is based on the genome sequence of the ancestral variant and is codon-optimized for expression in mammalian cells. However, SARS-CoV-2 has gone through a broad range of recombination, with the introduction of many mutations in its genome generating a vast array of variants. Most variants developed mutations in the S protein and the RBD. Such mutations may be of concern when developing serological assays as they may reduce the assay's sensitivity. Thus, the ELISA was also tested against other variants of concern that have resulted due to mutation of the ancestral variant to determine if it could also detect antibodies against different variants of concern.

2. Methods and Materials

2.1. Recombinant protein expression and purification

A plasmid expressing a soluble S protein of the SARS-CoV-2 isolate (GenBank MN908947.3) was kindly supplied by colleagues from Oxford University (Amanat et al., 2020). The vector map of the plasmid can be found in Appendix A. The nucleotide sequence encoding the S protein was codon-optimized for expression in mammalian cells. Briefly, the soluble version of the spike protein, including a C-terminal thrombin cleavage site, T4 foldon trimerization domain, and hexahistidine tag, was cloned into mammalian expression vector pCAGGS. The protein sequence was modified to

remove the polybasic cleavage site (RRAR to A), and two stabilizing mutations were introduced (K986P and V987P; wild-type numbering). An aliquot of SARS-CoV-2 S plasmid construct was transformed into One Shot Top 10 chemically competent *Escherichia coli* cells using the heat shock method. A single colony was selected, cultured, and scaled up. The overnight culture was purified using the QIAGEN plasmid maxi kit Qiagen, USA) according to manufacturer instructions. The purified plasmid DNA was eluted in nuclease-free water, and its concentration was measured using a Nanodrop spectrophotometer (Thermo Scientific, USA).

2.2. Mammalian cells

2.2.1. Cell maintenance

Human embryonic kidney cells (HEK-293) (ATCC® CRL1573) (ATCC, Manasses, United States of America (USA)) were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, United States) supplemented with 10% fetal bovine serum (FBS) (Lonza, Switerland), 1% L-glutamine (L-glut) (Lonza, Switzerland), 1% penicillin/streptomycin (Lonza, Switzerland), and 1% non-essential amino acids (NEAA) until they reached 80 to 90% confluency. When cells were 90% confluent, the growth media was removed, and the cells were washed twice with phosphate buffered saline (PBS) to remove serum traces. The cells were detached using trypsin, resuspended in growth media, and then cultured at 37°C in a humid environment until reaching 80-90% confluency again.

2.2.2. Transfection experiment

SARS-CoV-2 S plasmid was transfected into HEK-293 cells using Lipofectamine™ 3000 (Invitrogen, USA) according to the manufacturer's instructions in T75 cell culture vented flasks. Briefly, HEK-293 cells were seeded until 80-90% confluent. Plasmid DNA-lipid complexes were prepared by combining 28µL LipofectamineTM 3000 reagent with 938µL serum-free medium (Invitrogen, USA), supplemented with 1% NEAA, 1% L-glut, and 1% penicillin/streptomycin. For the master mix, 2 µg plasmid DNA was diluted in 938µL serum-free media, mixed with 7µL P3000™, and added to Lipofectamine™ 3000 in a 1:1 ratio and incubated at room temperature for 15 minutes. After incubation, the DNA-lipid complexes were added to HEK-293 cells and incubated for 72 hours at 37°C in a humid environment.

2.2.3. Confirmation of transfection by immunofluorescent assay

The expression of SARS-CoV-2 S protein in HEK-293 cells was confirmed using immunofluorescence assay (IFA). Transfected cells were spotted overnight and fixed onto a ten-well microscope slide in methanol: acetone solution for 20 minutes at 20°C. After fixing, cells were incubated with a primary antibody, serum from a COVID-19-positive individual, followed by a secondary antibody conjugated to fluorescein isothiocyanate (FITC) for 30 minutes each at 37°C. After washing with PBS (phosphate buffered saline) and mounting with glycerol mounting media (Euroimmun, Germany), the cells were examined under a Nikon fluorescent microscope, and bright green fluorescence indicated the expression of the SARS-CoV-2 S protein.

2.3. Purification of His-tagged protein

The recombinant expressed S protein with a 6x Histidine (His) tag was purified using Protino® Ni-TED (Macherey-Nagel, Germany) according to the manufacturer's instructions. A total of six T75 vented cell culture flasks were used for each transfection experiment. Briefly, the Protino column was first equilibrated using 4 mL of LEW buffer (supplied in the kit), and the S protein-containing media was loaded and allowed to flow through. The column was washed twice using 4 ml LEW buffer, and three fractions were collected from the column by adding 3 mL of elution buffer (supplied in the kit). The eluted S protein was concentrated using a 10 kDa Amico Ultra Centrifugal filter unit at 4000 x *g,* until about 500 µL of the protein remained in the unit. The protein concentration was then determined using the Qubit™ protein assay kit (Invitrogen, USA). The purified protein was stored at -80°C before use.

2.4. Serum samples

To validate the two in-house assays, ELISA and IFA, 89 serum samples were collected from convalescent patients who tested polymerase chain reaction (PCR) positive for SARS-CoV-2 between March and October 2020 and were tested for anti-SARS-CoV-2 IgG antibodies. Of the 89 PCR-positive samples, 48 samples were randomly selected and tested using two commercial assays, Elecsys® Anti-SARS-CoV-2 (Roche Diagnostics GmbH, Mannheim, Germany) and COVID-19 IgG/IgM Rapid Test cassette (Zhejiang Orient Gene Biotech Co., Ltd, Zhejiang, China). A panel of 100 residual diagnostic samples were obtained from the Division of Virology, National Health Laboratory Services (NHLS) before the pandemic and used as negative

controls. Permission was obtained from the NHLS business manager for the use of samples. A further 62 samples were collected from different COVID-19 waves between November 2020 and October 2021. The samples were used to determine if the assay could detect IgG antibodies against different circulating SARS-CoV-2 variants. Table 2.1 summarises the collection of samples. All patients completed informed consent documents and approval for the study was obtained from the University of the Free State Health Sciences Research Ethics Committee (UFS-HSD2020/0595/2605) and the Environment and Biosafety Ethics Committee (UFS-ESD2020/0072).

Table 2.1: Cohort of patients and negative controls

Collection Period	Number of serum samples	Purpose	Assay performed
Pre-pandemic (prior to January 2020)	100	Negative control for validation	In-house ELISA and IFA
March–October 2020	89	Validation	In-house ELISA and IFA
	48* (Subset of 89 serum samples)	Validation/ Comparison	In-house ELISA and IFA Elecsys® Anti-SARS-CoV-2 ELISA & COVID-19 IgG/IgM Rapid Test cassette
November 2020–October 2021	62	Performance testing	In-house ELISA and IFA

2.4.1. Indirect immunofluorescence assay (IFA)

IFA was performed on patient serum to confirm the presence of IgG antibodies against SARS-CoV-2, detailed in section 2.2.3. Transfected cells were incubated with patient serum diluted 1:10 in PBS at 37ºC in a humidified incubator for 30 minutes. Post-incubation, the cells were washed thrice with PBS and incubated with goat anti-human IgG (Invitrogen, United States) secondary antibody conjugated to FITC, diluted 1:20 in Evans blue, at 37ºC in a humidified incubator for 30 minutes. Following incubation, the IFA slide was washed three times, air-dried, mounted using glycerol mounting media, and covered with a coverslip. The slides were examined under a fluorescent microscope (Nikon Eclipse N*i*) to detect positive SARS-CoV-2 antibody fluorescence.

2.4.2. Enzyme-linked immunosorbant assay (ELISA)

ELISA was developed using purified supernatant from HEK-293 cells transfected with SARS-CoV-2 S protein. A 96-well maxisorp ELISA plate (Nunc MaxiSorp, Thermo Scientific) was coated with a 100µL of 3µg/mL of purified S antigen diluted in carbonate buffer and incubated overnight at 4ºC. The following day, the plate was washed three times, blocked for an hour with 100µL of 10% skimmed milk, and incubated at 37ºC in a humidified incubator. Post-incubation, the plate was washed three times with 0.1% phosphate buffered saline with tween 20 (PBST). Each serum sample was tested at 1:50 diluted in 2% skimmed milk. Each plate included three controls, negative, high positive, and low positive, tested in duplicate. The plate layout can be seen in Appendix B. A total of 100µL of the samples/ controls was added to each well coated with S antigen or mock antigen (carbonate buffer), and the plate was incubated at 37ºC for 2 hours. Post-incubation, the plate was washed six times with 0.1% PBST, and positive reactors were detected with a secondary antibody, goat anti-human IgG (Invitrogen, United States) conjugated to horseradish peroxidase (HRP) diluted 1:2000 of 2% skimmed milk and incubated at 37°C for an hour. The ELISA plates were then washed three times with 0.1% PBST. Subsequently, 100µL substrate reagent, 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS), was added to each well, and optical density (OD) at 405 nm was measured at 10 minutes post-incubation. Normalized results were obtained by the difference between the OD of the purified recombinant S protein-coated plate and the carbonate-coated plate (mock antigen). The results were represented as net OD and percentage positive (pp).

3. Results

3.1. In-house enzyme-linked immunosorbent assay

3.1.1. Repeatability of the assay

The repeatability of the assay was evaluated by testing one negative and one positive serum for SARS-CoV-2 IgG. Samples were run in triplicate on three different plates daily to a total of 60 replicates. Repeatability was calculated by taking inter-assay and intra-assay variance into account. Figure 3.1 shows the net OD obtained for positive and negative control at 10 minutes. The internal quality control data was generated by determining the mean +/- standard deviation (SD) of the 60 replicates of the controls.

Table 3.1 summarizes the results, including the mean OD and SD obtained for the controls and the upper and lower OD obtained for the negative and positive controls. The upper control limit (UCL) and the lower control limit (LCL) provide limitations of the in-house ELISA based on the controls falling within the accepted UCL and LCL (Paweska et al., 2003).

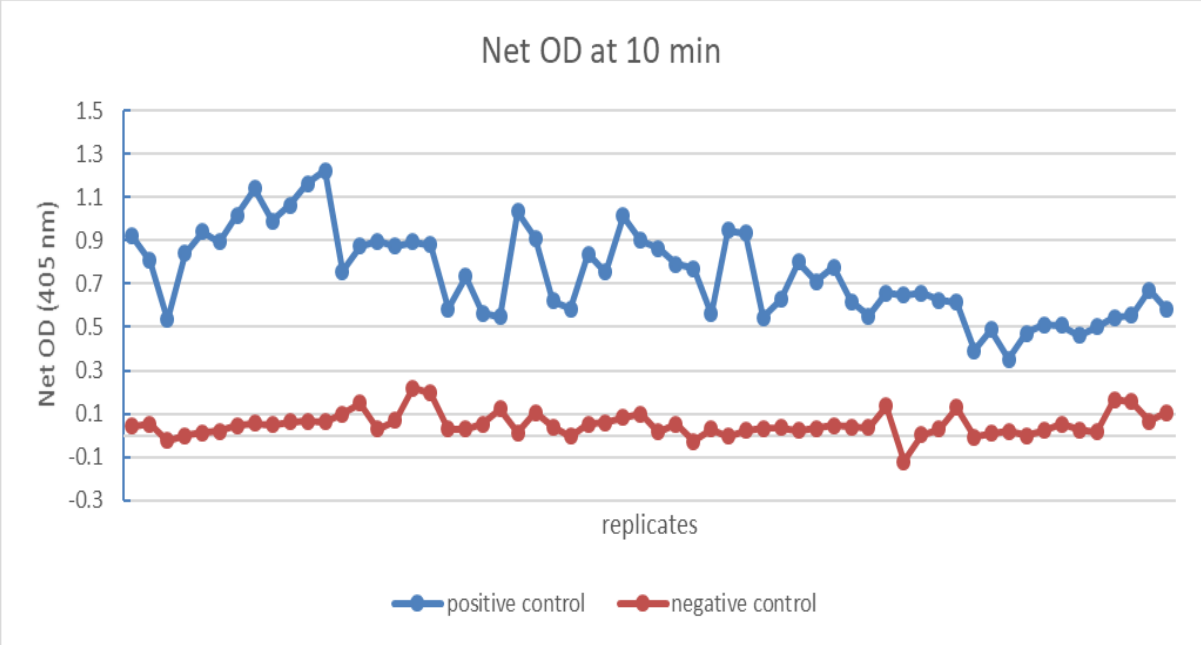

Figure 3.1: Repeatability of the assay. Repeatability was evaluated by testing the negative and positive control samples in triplicates on three different plates to a total of 60 replicates.

Table 3.1: Internal quality control data obtained for IgG in-house ELISA

	Mean optical density (OD_{405})	**Standard deviation**	**Upper control level OD_{405}**	**Lower control level OD_{405}**
Optical density (OD_{405}) C++	0.751	0.199	1.149	0.353
Optical density (OD_{405}) C-	0.0504	0.056	0.162	-0.062

C++ positive control, C- negative control

3.2. Determining the cutoff for the assay

The cutoff value for the IgG ELISA was determined using 89 convalescent sera collected from patients who tested positive for COVID-19 by PCR. The time of collection post-symptom onset varied among the patients; for others, it was not

included. Samples were collected from day 2 to 94 post-symptom onset. A set of 100 residual diagnostic samples, collected before the beginning of the pandemic, were used as negative control samples. ELISA results were interpreted by measuring the OD at 405 nm. Results were obtained by the difference between the OD of the purified recombinant S protein-coated plate and the carbonate-coated plate (mock antigen). Results were represented as net OD and then converted to pp using equation 1 (Paweska et al., 2007). Normalized results were expressed as pp of the positive control to account for variability that may have occurred between runs. High anti-S antibodies were detected in patients compared to samples collected before the beginning of the pandemic. Row data for the positive samples and negative panel can be found in appendices C and D, respectively. Notably, low levels of anti-S IgG were observed in some patients. Serum from these patients was collected during the first few days post-onset of symptoms. A total of 82/89 patient samples tested positive for IgG antibodies, giving a positive predictive value of 92%. All samples from the negative panel tested negative except four samples that cross-reacted, testing positive. Figure 3.2 shows the ELISA results obtained, expressed as percentage positive. The OD raw data and the pp values from the positive cohort and the negative panel can be found in appendices C and D, respectively.

$$\text{Percentage positive (pp)}(\%) = \frac{\text{sample optical density (OD405)}}{\text{C++ net OD405}}\ 100$$

Equation 1: Percentage positive calculation

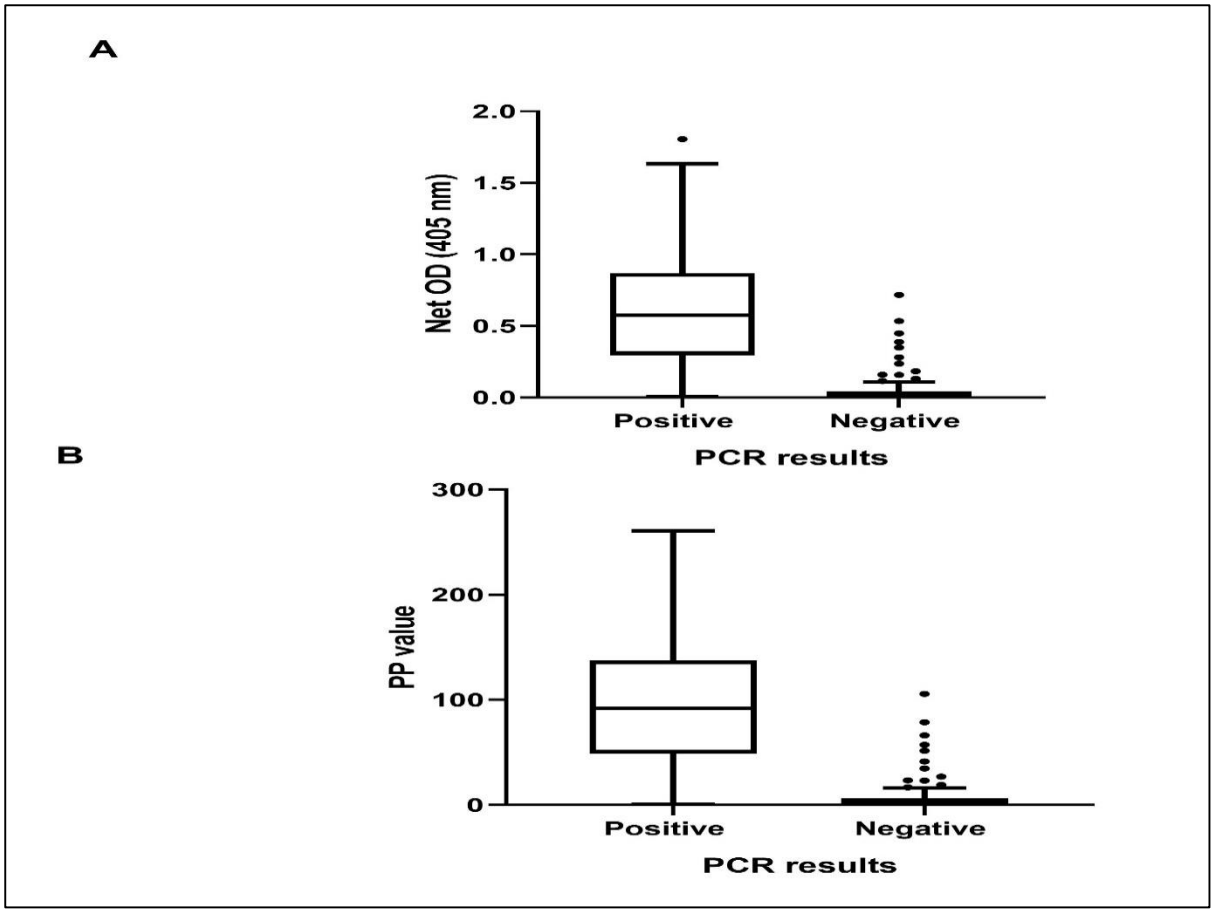

Figure 3.2: IgG response in PCR confirmed COVID-19 positive samples (n = 96) and COVID-19 negative samples (n = 100). A: Optical density readings. B: percentage positive values

The study evaluated the SARS-CoV-2 IgG antibody response in patients who had tested positive for SARS-CoV-2 by PCR. A panel of 100 samples collected before the pandemic was used as a negative control. A two-graph-receiver operating characteristic curve (TG-ROC) (Figure 3.3) was constructed against the percentage positive data of the collected samples to determine the cutoff value of the assay to differentiate between negative and positive samples. Samples acquired before the pandemic were labeled true negatives, and convalescent samples from patients with PCR-confirmed COVID-19 infection were true positives. A sensitivity of 96% and specificity of 92% were obtained for the IgG ELISA. A cutoff percent value of 30% was considered suitable to differentiate positive samples from negative using the TG-ROC graph. Figure 3.3 shows the TG-ROC with the sensitivity and specificity of the assay plotted against percentage positive. A pp value between 30% and 56% represented an indeterminate outcome; a pp value below 30% was deemed negative.

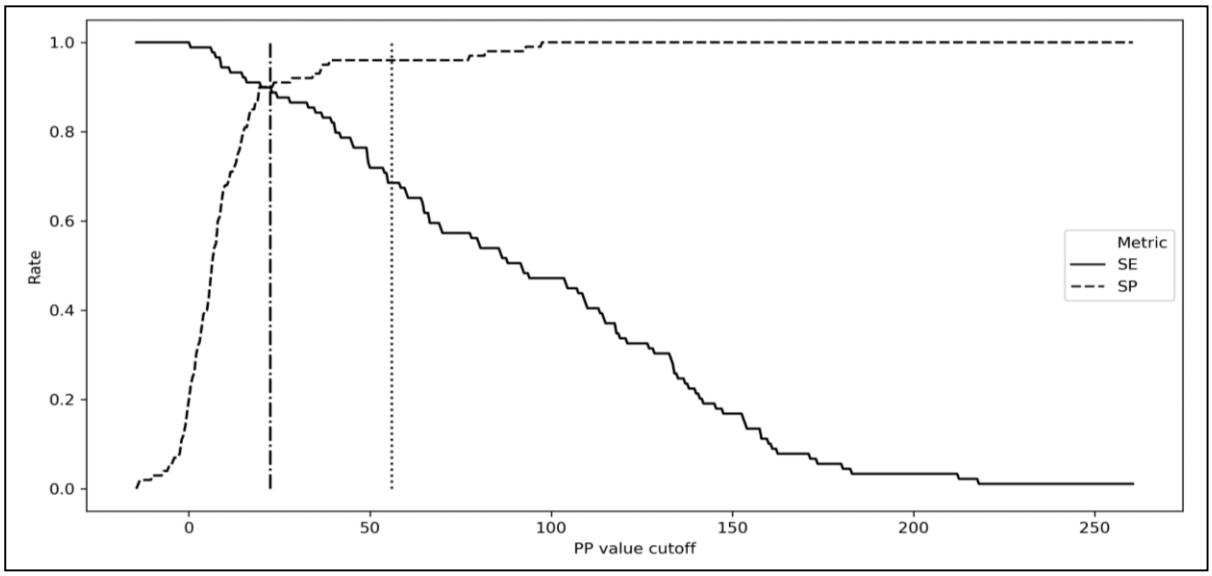

Figure 3.3: Two-graph-receiver operating characteristic curve showing the sensitivity and specificity of the IgG in-house ELISA at the different cutoff values. A 100 pre-pandemic samples and 89 COVID-19 PCR-positive samples were used for analysis. PP percentage positive, SE sensitivity, SP specificity.

3.3. Immunofluorescent assay

SARS-CoV-2 in-house IFA slides were developed from transfected cells expressing the SARS-CoV-2 S protein. Serum samples were reacted with FITC labeled goat anti-human IgG secondary antibody, and the presence of fluorescence identified seropositive samples, while negative samples produced no fluorescence. Out of 89, 81 samples tested positive for SARS-CoV-2 IgG antibodies with the in-house IFA, giving a positive predictive value of 91%. Figure 3.4 shows fluorescence in the positive IgG samples (figure 3.4A), whereas no fluorescence was observed using any negative-control sample (figure 3.4B) with the IFA. Table 3.2 summarizes the positive predictive values obtained by the two in-house assays.

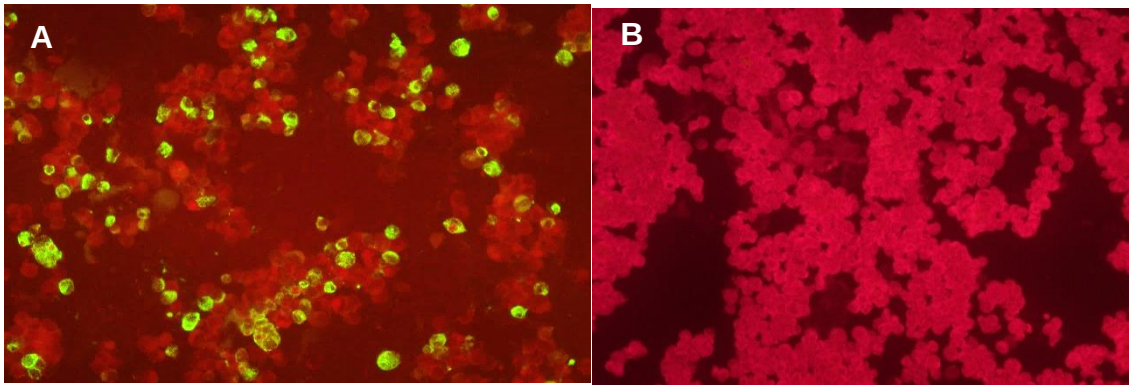

Figure 3.4: In-house immunofluorescence assay against SARS-CoV-2. Transfected cells reacted with human serum as the primary antibody, and goat anti-human IgG conjugated to fluorescein isothiocyanate (FITC) as the secondary antibody. Fig 3.4A IgG positive SARS-CoV-2 sample. Fig 3.4B IgG negative for SARS-CoV-2.

Table 3.2: Detection of antibodies by ELISA and IFA

	IFA positive	**IFA negative**
ELISA positive	78	4
ELISA negative	3	4

Samples from COVID-19-confirmed patients were collected between day 2 and 94 post-onset of symptoms. However, there were patients for whom data was unavailable regarding their onset of symptoms. As depicted in Table 3.2, seven samples from the COVID-19 confirmed patients tested negative with the ELISA and eight with the IFA. These samples were collected between day 2 and 6 post-symptom onset, and previous studies have shown that IgG antibodies against SARS-CoV-2 are usually detected one week post-symptom onset (Sun et al., 2020), explaining why the assay may have detected the samples as negative for antibodies as blood was collected before the patients mounted an immune response against the virus.

3.4. Comparison of the in-house assay with commercial assays

A total of 48 randomly selected PCR-positive samples were selected and tested using two commercial assays, the lateral flow assay, and the Roche assay, and compared to the in-house ELISA. A summary of the results can be seen below in Table 3.3 and Table 3.4. Comparison between the ELISA and the LFA resulted in 44 samples testing positive with both the ELISA and LFA, a single sample testing negative with both, and a variation with three samples. Comparison between the ELISA and the Roche assay

resulted in 42 samples testing positive with both assays, one sample testing negative with both assays and a variation with five samples. This resulted in a PPV of 95.8% for the ELISA, 93.9% for the LFA, and 89.6% for the Roche assay.

Table 3.3: Detection of IgG antibodies by ELISA compared between LFA and Roche assay

	LFA positive	LFA negative	Roche positive	Roche negative
ELISA positive	44	2	42	4
ELISA negative	1	1	1	1

Table 3.4: Detection of IgG antibodies by LFA compared to Roche

	LFA positive	LFA negative
Roche assay positive	42	3
Roche assay negative	1	2

The four samples from the negative panel that cross-reacted when tested with the ELISA were further tested with the LFA. The results obtained with the ELISA may be due to the presence of antibodies directed against antigens from other sources, including seasonal coronaviruses that may have displayed cross-reactivity with SARS-CoV-2 proteins. The outlier negative control samples that tested positive by ELISA tested negative with the LFA, showing the samples had no antibodies against SARS-CoV-2.

3.5. Sample collection from subsequent waves

A total of 62 additional samples from different waves were collected between June 2020 and September 2021 to determine if the in-house assay could detect different SARS-CoV-2 variants. All patients had a positive PCR test at the time of sample collection. Figure 3.5 summarizes sample collection, participants' vaccination status, and the circulating variants. The data for variant circulation was retrieved from the National Institute for Communicable Diseases (NICD) surveillance reports (https://www.nicd.ac.za/diseases-a-z-index/disease-index-covid-19/surveillance-reports/national-covid-19-daily-report/). The surveillance reports helped estimate the variants that were circulating at the time of sample collection. This suggests that the first wave continued from June 2020 to August 2020. The second wave of infections

increased in November 2020 and declined at the beginning of February 2021. The predominant variant circulating during the second wave was the Beta variant. The third wave, due to the Beta and the Delta variant, was between May and September 2021. Samples were collected during three different waves, with 44/62 patients vaccinated. Of the vaccinated, 14 received the Johnson and Johnson's Janssen vaccine, 29 received the Pfizer-BioNTech (COMIRNATY) vaccine, and one received the Vaxzevria vaccine (Astra-Zeneca). The remaining 18 participants were unvaccinated. Briefly, all samples were tested using the in-house IgG IFA and ELISA. All samples tested positive for SARS-CoV-2 IgG antibodies with the IFA and ELISA. The raw OD values and the pp data for the tested samples can be found in Appendix E. Figure 3.6 shows the level of IgG antibodies obtained by the different patients. A cutoff value of 30% was used to differentiate between negative and positive samples. The assays were developed using the sequences of the S protein of the SARS-CoV-2 ancestral strain. Therefore, it was essential to assess if the assays could be used to test for IgG antibodies against other variants. Despite the vast array of mutations on the S glycoprotein that lead to the different variants, the assay could still detect IgG antibodies of the circulating variants. The results indicate that the assay can be used to test for SARS-CoV-2 IgG antibodies against other variants.

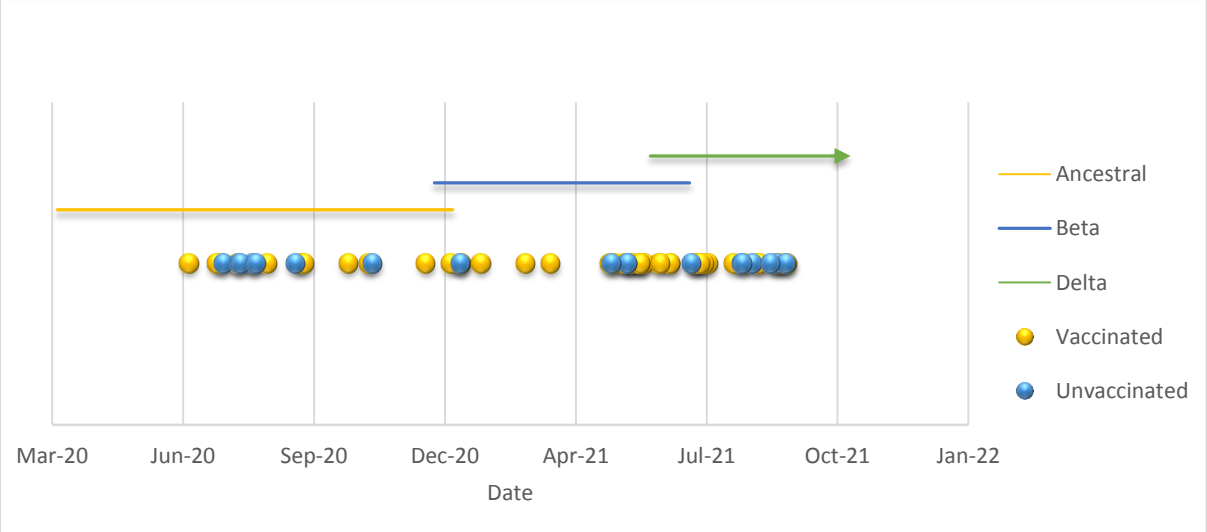

Figure 3.5: Representation of sample collection, the circulating variant, and whether the patients were vaccinated or unvaccinated.

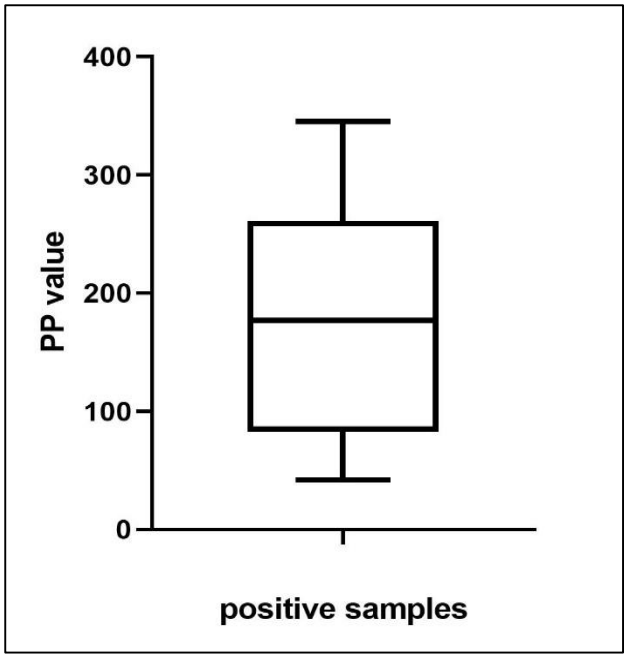

Figure 3.6: IgG antibody levels from patients infected with different variants of SARS-CoV-2. Additional samples were collected from 62 patients to determine if the in-house assays could detect IgG antibodies against other SARS-CoV-2 variants.

4. Discussion

The global spread of SARS-CoV-2 has resulted in substantial morbidity, mortality, and social disruption. Upon infection with SARS-CoV-2, the host elicits a humoral response by producing various classes of antibodies, including IgA, IgM, and IgG (Lapuente et al., 2023), from plasma cells, providing immunological evidence of exposure to the virus. Serological assays are a key tool that can be used to define exposure to the virus by detecting antibodies. Several serological assays based on lateral flow, chemiluminescence, or immunosorbent assays have been developed for the detection of antibodies against SARS-CoV-2 with varying sensitivities and specificities (Montesinos et al., 2020; Padoan et al., 2020; Tré-Hardy et al., 2021). Some assays are commercially available for testing antibody responses against SARS-CoV-2, but these assays can be costly. Thus, there is a need for the development of in-house assays. Current studies suggest that cell-mediated and humoral immunity play a protective role in SARS-CoV-2 infection, with the S and N proteins being highly immunogenic, with antibodies providing some immunity for a period of time (Shen et al., 2020).

It is important to evaluate and validate new serological assays to demonstrate their reliability and accuracy within the population for which the assays will be used. Many serological assays have emerged since the pandemic that described the use of recombinant protein for developing serological assays with high sensitivity and specificity (Liu et al., 2020; Muench et al., 2020). In this study, SARS-CoV-2 recombinant S protein was successfully transfected into HEK-293 mammalian cells. The expressed protein was used to develop the in-house IFA and ELISA to detect anti-S IgG antibodies in patients infected with SARS-CoV-2. The in-house IgG IFA and ELISA were developed and validated using PCR-positive SARS-CoV-2 samples collected from the South African population and a panel of negative samples collected prior to the pandemic. The assay's sensitivity and specificity were determined. ELISA results were interpreted by measuring the OD at 405nm and then converted to pp of the positive control to account for variability that may have occurred between runs, as the OD value may be influenced by different factor on daily bases including the ambient temperature, incubation times and even photometric instrumentation. A cuttoff percentage of 30% was considered accurate to differentiate between negative and positive samples using a TG-ROC. The two in-house assays showed high sensitivity and specificity. Samples from COVID-19 confirmed cases resulted in 92% tested positive predictive value (PPV) for the ELISA and 91% PPV for IFA. The samples that tested negative may have been due to low detectable antibodies as these samples were collected within the first few days off infection. Other studies have shown that some SARS-CoV-2 patients recover without the development of antibodies (Huynh et al., 2021).

In this study, 4/100 samples from the negative panel showed reactivity to the SARS-CoV-2 S protein, resulting in an overall specificity of 96%. The same samples that cross-reacted were tested using the COVID-19 IgG/IgM Rapid Test cassette, and none tested positive for SARS-CoV-2 antibodies, suggesting that the samples that displayed cross-reactivity may have been from patients infected with seasonal coronaviruses or other related viruses that may share similar epitopes. This is one of the main disadvantages of serological assays, the potential of the test yielding false-positive results. It is essential to develop serological assays with high specificity to reduce the number of false-positive results. One of the leading causes of false-positive results is cross-reactivity with seasonal coronaviruses and other analytes.

The in-house assays were based on the S protein of the ancestral variant of the virus but were able to detect IgG antibodies against other SARS-CoV-2 variants. However, the assay could still detect IgG antibodies when testing samples for patients infected with other SARS-CoV-2 variants, suggesting that epitopes on the SARS-CoV-2 S protein have not significantly changed, allowing for the detection of antibodies against other variants. This study demonstrated that the recombinant S in-house IFA and ELISA could be an essential method for COVID-19 surveillance, with high sensitivity, as it can also detect other SARS-CoV-2 variants.

Additionally, the performance and accuracy of the in-house ELISA was evaluated and compared to two commercially available South African Health Products Regulatory Authority (SAPHRA) approved assay, the Elecsys® Anti-SARS-CoV-2 and the COVID-19 IgG/IgM Rapid Test cassette using a cohort of randomly selected samples. The Elecsys® Anti-SARS-CoV-2 assay is based on the N protein, while the COVID-19 IgG/IgM Rapid Test cassette is based on the S protein. The rapid lateral flow test demonstrated a higher sensitivity to the Elecsys®, although studies have previously shown that lateral flow assays are usually less sensitive than other serological assays. However, the difference in target proteins between the two assays may also be a contributing factor. Studies have shown that assays directed against the S protein are more sensitive than those against the N protein. This may be due to the relatively high sensitivity and early response to the S antigen compared to the N antigen in patients with COVID-19 (Liu et al., 2020). The in-house IgG ELISA results showed comparable performance levels with a slightly higher PPV in detecting IgG antibodies for SARS-CoV-2 patients to the two commercial assays. One limitation was the small sample size for the comparison, as a limited number of tests were available.

These results suggest that both the in-house ELISA and IFA can be used for surveillance and testing of SARS-CoV-2 IgG antibodies, especially for the analysis of serum samples of patients taken one week post-onset of symptoms. These results support the potential use of the assays for identifying past exposure to SARS-CoV-2 in the South African population.

Authors Contribution

Litabe M: Investigation, Methodology, Data curation, Formal analysis. van Vuuren C: Investigation, Resources. Bester PA: Data curation, Formal analysis, Writing– review & editing. Goedhals D: Investigation, Resources, Writing– review & editing. Potgieter S: Investigation, Resources. Frater J: Investigation, Resources, Writing– review & editing. Thompson C: Investigation, Resources, Writing – review & editing. Wright D: Investigation, Resources, Writing– review & editing. Lambe T: Investigation, Resources. Gupta S: Investigation, Resources. Brink M: Investigation, Resources. van Jaarsveldt D: Investigation, Resources. Burt FJ: Conceptualization, Methodology, Writing – original draft, Writing– review & editing, Supervision, Project administration, Funding acquisition.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 4

Longitudinal assessment of IgG and neutralizing antibody responses in COVID-19 recovered patients in the Free State, South Africa

Matefo Litabe[1], Philip Bester[2], Felicity Burt [1,2]

1. Division of Virology, Faculty of Health Sciences, University of the Free State, Bloemfontein, South Africa
2. Division of Virology, National Health Laboratory Service, Bloemfontein, South Africa

Abstract

The recent severe acute respiratory coronavirus 2 (SARS-CoV-2) pandemic was responsible for more than 767 million cases and an estimated 6.9 million deaths. Understanding the kinetics and duration of antibody responses contributes towards understanding the longevity of immunity to SARS-CoV-2. This study aimed to investigate the duration of anti-S immunoglobulin (Ig)G antibodies using serological assays and the duration and cross-neutralization capacity of neutralizing antibodies against the Ancestral strain, Delta, and Omicron over time. Serum samples were collected at intervals from 100 convalescent patients infected with different variants and some who had received vaccine. Sera were tested for anti-S IgG antibodies and neutralizing antibodies against Ancestral strain, Delta, and Omicron variants. IgG antibody levels and neutralizing antibodies against each variant were significantly higher early after infection, gradually decreasing but remained detectable. IgG antibody was detected in 85% of sera up to 2.5 years post-infection, and neutralizing antibodies against Ancestral, Delta, and Omicron remained detectable in 85%, 81%, and 90% of samples. The results indicate that while IgG and neutralizing antibodies waned, a significant proportion of individuals maintained detectable levels even 24 months post-infection. Vaccinees had significantly higher neutralizing antibody levels, showing that vaccination has an important role in boosting immunity.

1. Introduction

Severe acute respiratory coronavirus 2 (SARS-COV-2), a member of the *Coronaviridae* family, emerged in December 2019 and rapidly spread worldwide to

become a major pandemic, with substantial morbidity, mortality, and social disruption. According to the World Health Organisation (WHO), there were over 767 million recorded cases by the beginning of June 2023 and an estimated 6.9 million deaths as a result of SARS-CoV-2 worldwide (https://covid19.who.in)

Ribonucleic acid (RNA) viruses have the propensity to mutate due to the replication strategy of the virus, and the high rate of mutations for SARS-CoV-2 led to the emergence of multiple variants, of which five were identified by the WHO as variants of concern (VOC). These variants carry different mutations within the receptor binding domain (RBD), resulting in either increased transmissibility, virulence, or immune resistance. The Alpha (B.1.1.7) variant, first described in the United Kingdom, has 23 mutations in the spike protein. Although the impact of all the mutations is not known, RBD N501Y appears to increase the affinity of binding angiotensin-converting enzyme (ACE2) receptor, making the virus more infectious (Davies et al., 2021). The Beta lineage, first identified in South Africa, has eight mutations in the S protein, with three substitutions in the RBD (E484K, K417N, and N501Y), resulting in increased transmissibility and immune escape (Tegally et al., 2021). Initially identified in India, the Delta variant has unique mutations, including D614G, L452R, P681R, and T478K in the S protein, possibly enhancing transmissibility and immunity to existing neutralizing antibodies (Dhawan et al., 2022). The Omicron variant (B.1.1.529), first detected in November 2021 in Botswana, carries at least 32 mutations on the spike protein, with 15 occurring within RBD (Cao et al., 2022).

The immune correlates of protection are not clearly defined but are likely multifactorial, involving both the humoral and cellular immune responses. Humoral immunity to SARS-CoV-2 is acquired from natural exposure or vaccination and is purported to provide some protection against infection, reinfection, and severity of disease. Previous SARS-CoV-2 antibody studies in COVID-19-recovered patients show that most recovered patients develop detectable virus-specific antibodies within two weeks post-symptom onset. Patients with more severe SARS-CoV-2 disease develop higher neutralizing antibody titers (Li et al., 2020; Zhao et al., 2020), although the duration of detection responses beyond six months is not yet clear (Vanshylla et al., 2021). As the pandemic progressed, VOC emerged with mutations in the spike protein that contributed towards evasion from existing immune responses induced by natural

infection or vaccination and reduced neutralization titers *in vitro* (Greaney et al., 2021; Cao et al., 2022).

In an effort to control the pandemic, vaccine programs were implemented worldwide. Detectable humoral immunity was reportedly shown to wane with time, occurring within months post-vaccination and/or infection (Levin et al., 2021; Goldberg et al., 2022). Although protection likely involves both the humoral and cellular responses, understanding the kinetics of antibody response and duration of responses will contribute towards understanding the longevity of immunity to SARS-CoV-2. This study aimed to investigate the duration of anti-S IgG antibodies using serological assays and the duration and cross-neutralization capacity of neutralizing antibodies against the Ancestral strain, Delta, and Omicron over time.

2. Methods and Materials

2.1. Ethics and Biosafety

Approval for the study was obtained from the University of the Free State Health Sciences Research Ethics Committee (UFS-HSD2020/2001/2601-0005) and the University of the Free State Environment and Biosafety Ethics Committee (UFS-ESD2020/0181/22). All experiments using the infectious SARS-CoV-2 virus were performed in the biosafety level 3 (BSL3) laboratory at the Pathogen Research Laboratory, Division of Virology, at the University of the Free State.

2.2. Sample Collection and Processing

A cohort of 100 patients with PCR-confirmed SARS-CoV-2 infection between June 2020 and December 2021 were recruited for the study between March 2021 and January 2022. Patients were enrolled at different times after the onset of symptoms. Blood samples were collected at enrolment, and serum samples stored at -80 ºC. Eighty-two patients returned for a second bleed between July and September 2022. Sixty-two patients were available for collection of a third blood sample between January and March 2023. Written informed consent was obtained from each patient. The samples were tested for IgG antibodies using an in-house enzyme-linked immunosorbent assay (ELISA) and for neutralizing antibodies against the Ancestral strain, Delta, and Omicron variants of SARS-CoV-2 using a microneutralization assay.

2.3. In-house ELISA for detection of SARS-CoV-2 IgG

The ELISA was developed using recombinant S protein expressed in human embryonic kidney (HEK-293) cells, as previously described (Matefo et al., 2022). Briefly, unless stated otherwise, all volumes were 100µL/well, plates were incubated for one hour at 37°C and washed for 3 x 15 seconds with 0.1% Tween 20 in phosphate buffered saline (PBS) pH 7.0. Briefly, 96-well PolySorp microtitre plates (Nalge Nunc International Corporation, USA) were coated with 100µL of 3 µg/mL of purified recombinant SARS-CoV-2 S protein diluted in carbonate buffer pH 8.6, overnight at 4°C. Control wells contained carbonate buffer without recombinant antigen. Plates were washed and blocked with 200µL/well 10% skimmed milk/PBS. Serum samples were diluted 1:50 in 2% skimmed milk/PBS and added to antigen-coated wells and control wells. Duplicate positive (C++) and negative (C-) controls were included on each plate. Positive reactors were detected using anti-human IgG horseradish peroxidase (HRP) conjugate diluted 1:2000 (SeraCare Life Sciences Inc., Milford, USA) and 2,2'-azino di-ethyl-benzothiazoline-sulfonic acid peroxidase substrate (ABTS) (SeraCare Life Sciences Inc., Milford, USA). Plates were incubated for 10 minutes at room temperature (22-24°C) in the dark, and the optical density (OD) values were read at 405 nm. Net OD values were determined for each sample as follows: net OD = OD in wells with recombinant SARS-CoV-2 antigen minus OD in wells with no antigen.

OD values were normalized to account for variability by determining the percentage of positive values based on positive control serum (Paweska et al., 2007) as follows: (net OD of test sample/net OD of C++) × 100. A cutoff value of 30%, which differentiated positive samples from negative samples, was determined in the previous validation (Matefo et al., 2022).

2.4. Virus isolation

SARS-CoV-2 variants were isolated in h1299-hACE2-E3 cells, a human lung cell line overexpressing human ACE2 receptor (kindly donated by Professor Alex Sigal, African Health Research Institute, University of KwaZulu-Natal). Viruses were isolated from residual diagnostic nasopharyngeal swabs post-confirmation of SARS-CoV-2 using reverse-transcriptase polymerase chain reaction (RT-PCR). Briefly, samples were diluted 1:1 with maintenance media, Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, USA) supplemented with 2% fetal bovine serum (FBS), 2%L-glutamine

(L-glut), 1% non-essential amino acids (NEAA) and 1% antibiotics, 1% sodium pyruvate, and 1% (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and filter sterilized using a 0.2µM nylon filter (GVS North America, USA). A 75µL aliquot of the sample was inoculated on a confluent monolayer of cells in a T25 vented cell culture flask. Cells were incubated at 37ºC for 30 minutes to allow virus adsorption. Post-incubation, a 5mL aliquot of maintenance media was added to the flask, and cells were incubated at 37ºC in a CO_2 incubator with 5% CO_2. Cells were monitored daily for cytopathic effects (CPE). At the first sign of CPE, an aliquot of cell culture media was tested using the rapid SARS-CoV-2 antigen test card (Boson Biotech, China). Positive cell cultures were incubated until 90% CPE was observed, at which time the cells were freeze-thawed and clarified by centrifugation at 1200 *xg* for 5 minutes. Samples were stored at -80ºC before use. Confirmation of the isolates was performed using next-generation sequencing using published protocol of Oxford Nanopore Technology(Tyson et al. 2020) (https://protocols.io/view/ncov-2019-sequencing-protocol-v3-locost-bh42j8ye) .

2.5. Determination of tissue culture infectious dose 50 ($TCID_{50}$)

Tissue culture infectious dose 50 ($TCID_{50}$) was determined for each isolate by performing tenfold serial dilutions of SARS-CoV-2 virus stock using h1299-hACE2-E3 cells in flat-bottomed 96 well plates, from 10^{1} to 10^{-7}, in replicates of six. A 50µL aliquot of diluted virus was added to the 96 well-plate, followed by 100µL of $1x10^{5}$ h1299-hACE2-E3 cells. A 50µL aliquot of media was added to cells without the virus as a control. CPE was observed under the microscope over three days, and the $TCID_{50}$ was calculated using the Reed–Muench method (Reed & Muench, 1938; Ramakrishnan, 2016).

2.6. Virus micro-neutralization test

SARS-CoV-2 specific neutralizing antibody levels against Ancestral strain, Delta, and Omicron isolates were measured in duplicate with a microneutralization test in a BSL 3 laboratory. The control plate included a virus back titration and positive and negative controls in duplicate. The back titration was performed to confirm the correct virus test dose of 100 $TCID_{50}$. Briefly, the working dilution of the virus, 100 $TCID_{50}$, was back-titrated by preparing a tenfold serial dilution from 100 $TCID_{50}$ to 1 $TCID_{50}$. The diluted virus was inoculated onto h1299-hACE2-E3 cells in replicates of four per dilution and

examined daily for CPE. Briefly, the serum samples were heat-inactivated at 56°C for 30 minutes. A twofold serial dilution, from 1:10 to 1:640, of the samples was performed using RPMI 1640 supplemented with 10% FBS, 1% NEAA, 1% antibiotics, 1% sodium pyruvate, 1% HEPES, and 2% L-glut. Serum samples were incubated with 50 µL of 100 $TCID_{50}$ SARS-CoV-2 virus for 30 minutes at 37°C in a humidified CO_2 incubator. The final dilution of samples was 1:20 to 1:1280. Post-incubation $2x10^5$ cells of h1299-hACE2-E3 were added to the serum-virus mix and incubated at 37°C in a humidified CO_2 incubator. The sample and control plates were monitored daily for CPE. On day three, the neutralization endpoint was determined as the highest serum dilution, resulting in 50% inhibition of CPE.

2.7. Statistical analysis

Statistical differences between groups were assessed using the one-way Analysis of variance (ANOVA) test (https://www.statskingdom.com) with post-hoc Tukey's Honest Significant Difference (HSD) for multiple comparisons test. A $p<0.05$ was considered statistically significant.

3. Results

3.1. Cohort for investigating SARS-CoV-2 immunity and circulating variants

To investigate the duration of SARS-CoV-2 immunity, a cohort of 100 participants were recruited, and serum samples were collected from participants on three occasions. Initial blood samples were collected between 12 days and 13 months post-symptom onset. The gender and vaccine history of participants is summarized in Table 3.1. The cohort included 62/100 females and 38/100 males. A total of 64% were vaccinated against SARS-CoV-2, which included 40.6% (26/64) with Pfizer-BioNTech, 57.8% (37/64) with Janssen (Johnson & Johnson), and 1.6% (1/64) with Oxford/AstraZeneca. The vaccination program was introduced in SA in February 2021, hence the study had a mixed population of vaccinated and unvaccinated individuals. Disease onset was determined from the onset of self-reported symptoms or the date of positive RT-PCR test. Figure 3.1 shows the emergence and circulation of each variant in SA (https://www.nicd.ac.za/diseases-a-z-index/disease-index-covid-19/surveillance-reports/national-covid-19-daily-report/) and the date of symptom onset, or positive RT-PCR, was used to assume infecting variant. Thirty serum samples were collected from individuals with acute illness between August 2020 and November 2020, when the

Ancestral variant was circulating. A total of 56 samples were collected from individuals reporting acute illness between December 2020 and September 2021, when the circulation of Beta and Delta variants overlapped, and 14 samples were collected from participants with confirmed infections between December 2021 and January 2022 during the circulation of the Omicron variant. Follow-up samples were collected from 82/100 of the initial cohort between July 2022 and September 2022, which included samples collected 232 to 783 days post-symptom onset. Sixty two participants were bled on a third occasion, with samples collected 408 to 976 days post-symptom onset. The remaining participants were lost in the follow-ups as they either moved from Bloemfontein or withdrew from the study.

Booster vaccinations were documented for 35/82 and 2/62 individuals. Reinfections were recorded for 8/82 and 1/62.

Table 3.1: Gender and vaccine history of participants

	Vaccinated	**Unvaccinated**	**Vaccine history unavailable**
Gender n=100	Female: 39%(39) Male: 25%(25)	Female:18% (18) Male: 12%(12)	Female: 5% (5) Male: 1% (1)
Vaccine type n=64	Pfizer: 40.6% (26) J&J: 57.8% (37) AstraZenica: 1.6% (1)		
Booster vaccination Pfizer	2022: 42.7% (35/82) 2023: 3.2% (2/62)		
Reinfection 2022:8 2023: 1	2022: 62.5% (5) 2023: 100% (1)	2022: 37.5% (3)	

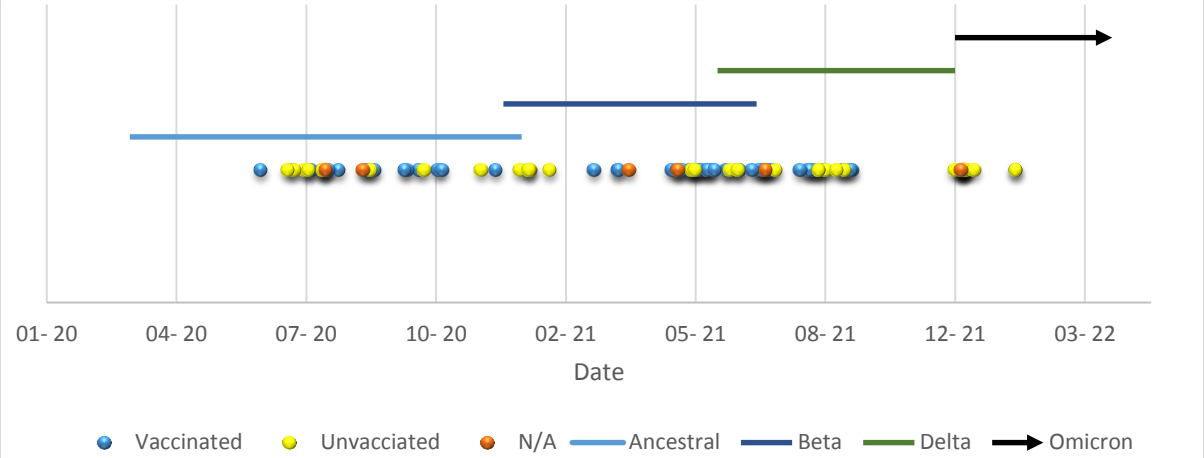

Figure 3.1: Variant circulation and vaccination status. A total of 100 participants were recruited for the study and grouped according to their vaccination status and likely variant of infection. The lines represent the variant that was circulating at the time of sample collection, and the colored circles represent the vaccination history of the individuals from which blood was collected.

3.2. IgG Antibody Kinetics

Serum samples were tested for IgG antibodies using in-house ELISA and for neutralizing antibodies using microneutralization assays to determine the duration of detectable antibodies against SARS-CoV-2. A total of 244 serum samples were collected from 100 individuals between March 2021 and March 2023, ranging between 12 days to 976 days post-symptom onset.

These samples were tested for IgG antibody using ELISA, and readings obtained from the ELISA were converted to percentage positive (pp) values to account for any variability between runs. The pp value was consistently determined using the same positive control, allowing comparison of samples, normalization of results, and used as an indication of antibody levels. A cutoff value of 30%, determined in a previous study, was used to differentiate positive samples from negative samples (Matefo et al., 2022). A total of 95/100 baseline samples tested positive for anti-S IgG antibody using ELISA, 79/82 samples were positive at the second bleed, and 53/62 samples were positive at the third bleed. Serum samples were grouped based on the time (days) the sample was collected after onset of symptom (or RT-PCR result), as follows: samples collected 1-180 days (63/244), 181-360 days (33/244), 361-540 days (73/244), 541-720 days (45/244), and 721 to 1000 days (30/244) (Figure 3.2). Using

pp values as an indicator of antibody levels, IgG antibody levels were significantly higher early after infection, 1-180 days, compared to 361-540 days ($p=6.94x10^{-3}$), 541-720 days ($p=6.28x10^{-6}$), and 721 to 1000 days ($p=1.81x10^{-7}$). Antibody levels were not statistically significant different in samples collected 1-180 days and samples collected 181-360 days (p=0.08) post onset. Antibody levels were significantly higher in the 181-360 days group compared to 541-720 days (p= 0.05), and 721 to 1000 days (p=0.03). Similarly, anti-S IgG antibody levels were significantly higher in the 361-540 days group compared to the 721-1000 day group (p=0.01). There was no statistical significance in antibody titers between samples collected 541-720 days and 721 to 1000 days (p=0.7). Hence, a gradual but significant decrease was shown over time despite possible reinfection and vaccination.

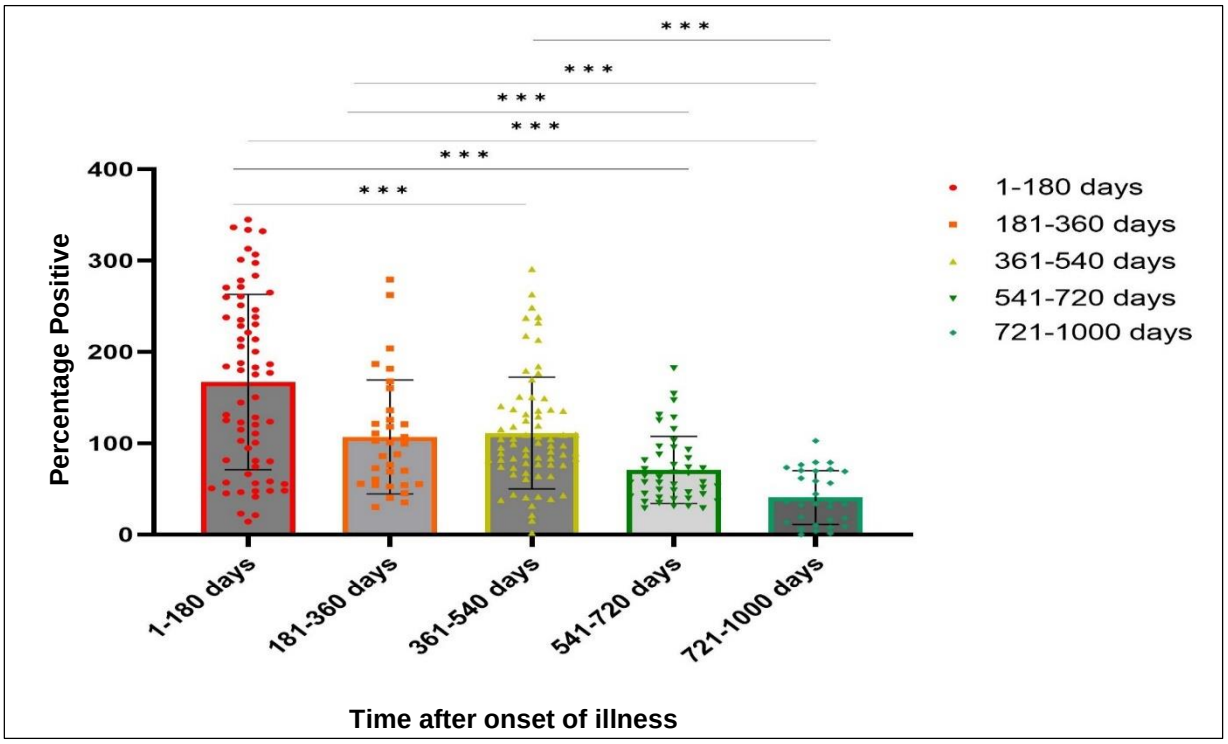

Figure 3.2: Antibody levels in SARS-CoV-2 patients. The level of IgG antibodies were measured using an ELISA in 244 samples collected between 2021 and 2023 at various times post-initial diagnosis. The results are represented as percentage positive (pp) of the positive control. ***P>0.05.

Figure 3.3 compares the antibody levels of participants that were vaccinated and those that were unvaccinated. Antibody levels against the SARS-CoV-2 S protein were significantly higher in the vaccinated group compared to the unvaccinated group in

samples collected between 1-180 days, 181-360 days, and 361-540 days, possibly boosted due to vaccination and additional exposure to the SARS-CoV-2 antigen. However, there was no statistical significance in IgG levels between vaccinated and unvaccinated individuals in the groups of 541-720 and 721-1000 days post-infection. An IgG antibody response remained detectable two years post-infection in 85.5% of participants who returned for the third bleed, and 11/16 samples collected between 721-976 days were negative. A total of 44/62 individuals that remained positive for IgG antibodies at the final draw had received vaccine. Overall, the results indicate that although IgG antibody levels may decline with time, the majority of individuals still had detectable titers at least 24 months post-acute infection.

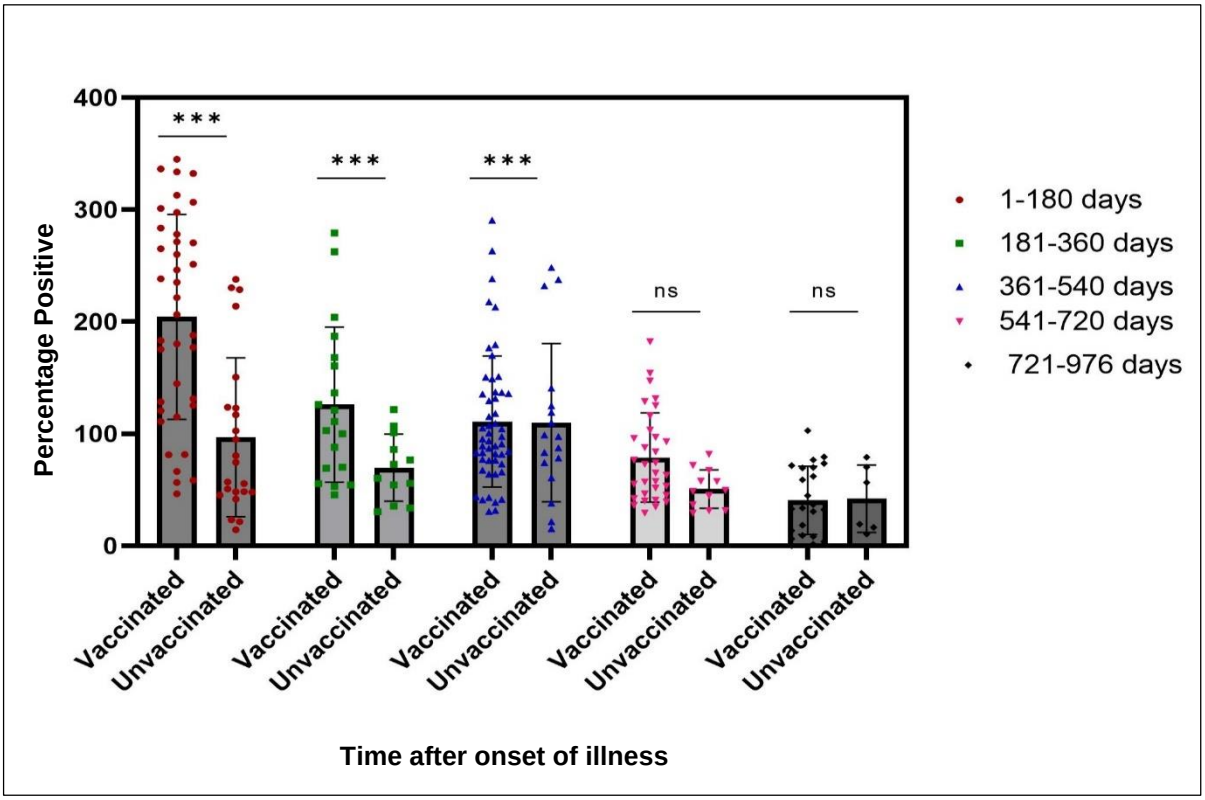

Figure 3.3: Comparison of antibody levels between vaccinated and unvaccinated participants over the collection period. Serum samples were collected between 12 and 976 days post-symptom onset and grouped based on time of collection post-symptom onset. ***P>0.05, ns not significant.

3.3. Kinetics of neutralizing antibodies by microneutralization assay

3.3.1. Isolation of virus

Residual diagnostic samples (nasopharyngeal swabs) from patients with confirmed SARS-CoV-2 using real-time RT-PCR and with a cycle threshold (Ct) of <30 were selected for virus isolation attempts. Samples were allocated a laboratory number (VBD) for identification, and the virus isolation was performed in the BSL 3 laboratory. CPE was observed between two and three days post-inoculation, and the cell lysate was harvested on day four. Low passage isolates were used for neutralization assays where possible to prevent mutations due to passaging through cells. All isolates were passaged minimally to minimize the introduction of mutations. Three isolates were available, representing each VOC circulating in South Africa, isolate-designated VBD 7/20 was an Ancestral strain, VBD 247/21 was a Delta variant, and VBD 137/22 was an Omicron variant. Virus isolates VBD 7/20 and VBD 247/21 were passaged in cells twice, and VDB137/22 was passaged once. The identification of the Ancestral isolate and VOC were confirmed using next generation sequencing (NGS).

The $TCID_{50}$ was calculated for each isolate as follows: VBD 7/20 log $TCID_{50}$ $10^{3.68}$, VBD 247/21 log $TCID_{50}10^{4.33}$, and VBD137/22 log $TCID_{50}$ $10^{3.6}$.

3.3.2. Comparison of neutralizing antibody titers against SARS-CoV-2 isolates

Neutralization antibody titers were quantified against the SARS-CoV-2 Ancestral strain, Delta, and Omicron variants. Samples were diluted two-fold from 1:20 to 1:1280, and the neutralizing antibody titer was determined as the reciprocal of the highest dilution inhibiting 50% CPE in cells. Table 3.2 shows the total number of serum samples that tested positive for neutralizing antibodies against each variant. Neutralizing antibody titers were compared at intervals after the onset of symptom against each variant (Figures 3.4 A and B) and were significantly higher in samples collected between 1-180 days post-infection compared to those collected between 721-1000 days post-infection (p=0.02). However, although not statistically significant, neutralizing antibody titers in samples collected at 721 to 1000 days did not appear to wane against Omicron (Figure 3.4 C), possibly due to reinfection with asymptomatic or milder illness not requiring testing. Omicron was reported to cause less severe disease (Christensen et al., 2022), and fewer restrictions on travel and daily life likely facilitated transmission.

Overall, the results show that neutralizing antibody titers against the Ancestral strain and Delta variant remain relatively stable for an extended period and then wane gradually before declining significantly.

Table 3.2: Total serum samples positive for neutralizing antibodies

	Baseline draw	1st follow-up	2nd follow-up
Ancestral strain	99/100	80/82	53/62
Delta variant	78/100	63/82	50/62
Omicron variant	72/100	77/82	56/62

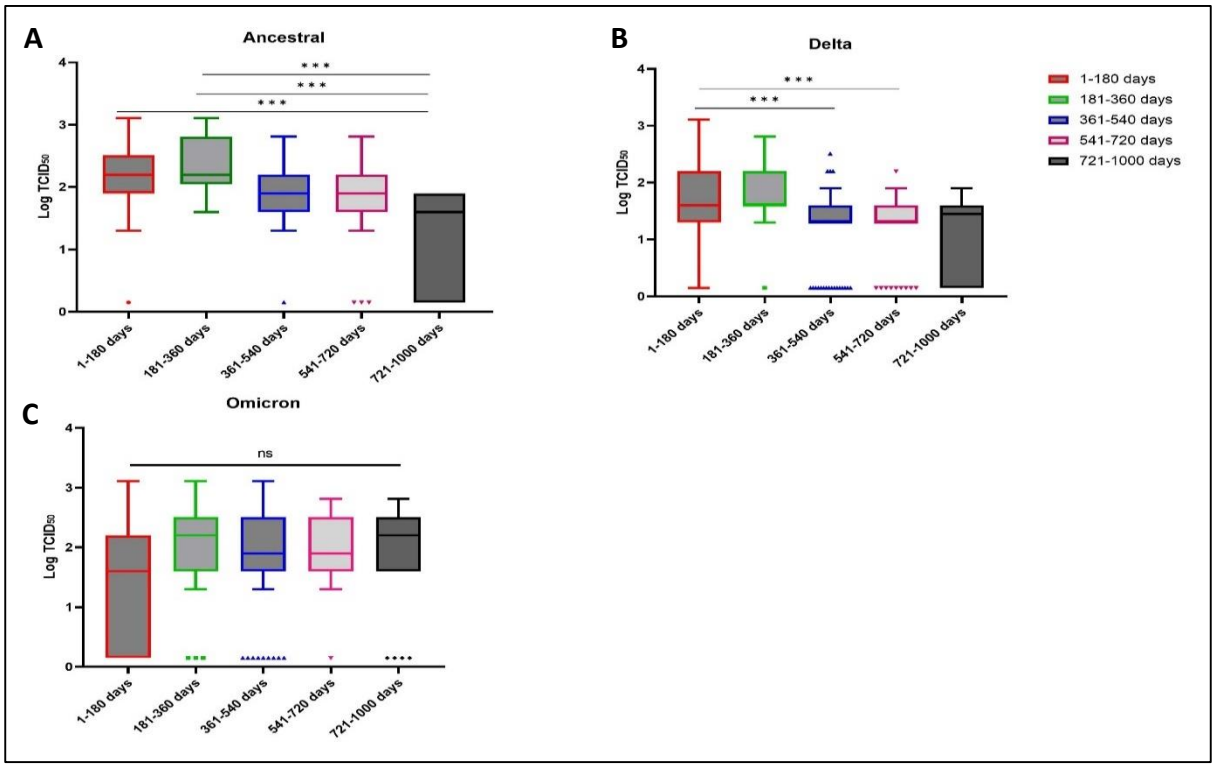

Figure 3.4: Comparison of neutralizing antibody titers in a total of 244 samples collected from 100 individuals between 2021 and 2023 at various times post-initial diagnosis against Ancestral strain, Delta, and Omicron variants. A. Ancestral strain. B. Delta variant. C. Omicron variant. ***P>0.05, ns not significant.

3.3.3. Comparison of neutralizing antibody titers between vaccinated and unvaccinated participants

Comparing vaccinated and unvaccinated individuals, a significant difference in neutralizing antibody titers was observed against the Ancestral strain in samples collected 1-180 days (p=0.01), 181-360 days (p=0.05) post-infection and no significant

difference in samples collected between361-540 days (p=0.2) and 541-720 days (p=0.82) and 721-980 days (0.43) post-infection (Figure 3.5A). Neutralizing antibody titers against the Delta variant were significantly higher in vaccinated participants compared to the unvaccinated in samples collected between 1-180 days post-infection (Figure 3.5B), with no statistical significance in neutralizing titers among the other groups. Neutralizing antibody titers were significantly higher in the vaccinated group compared to the unvaccinated group in samples collected between 181-360 days (p=0.03), 361-540 days (p=0.05), and 541-720 days (p=0.04) against the Omicron variant (Figure 3.5C). The results suggest that vaccination had an impact on the development of higher neutralizing antibody titers.

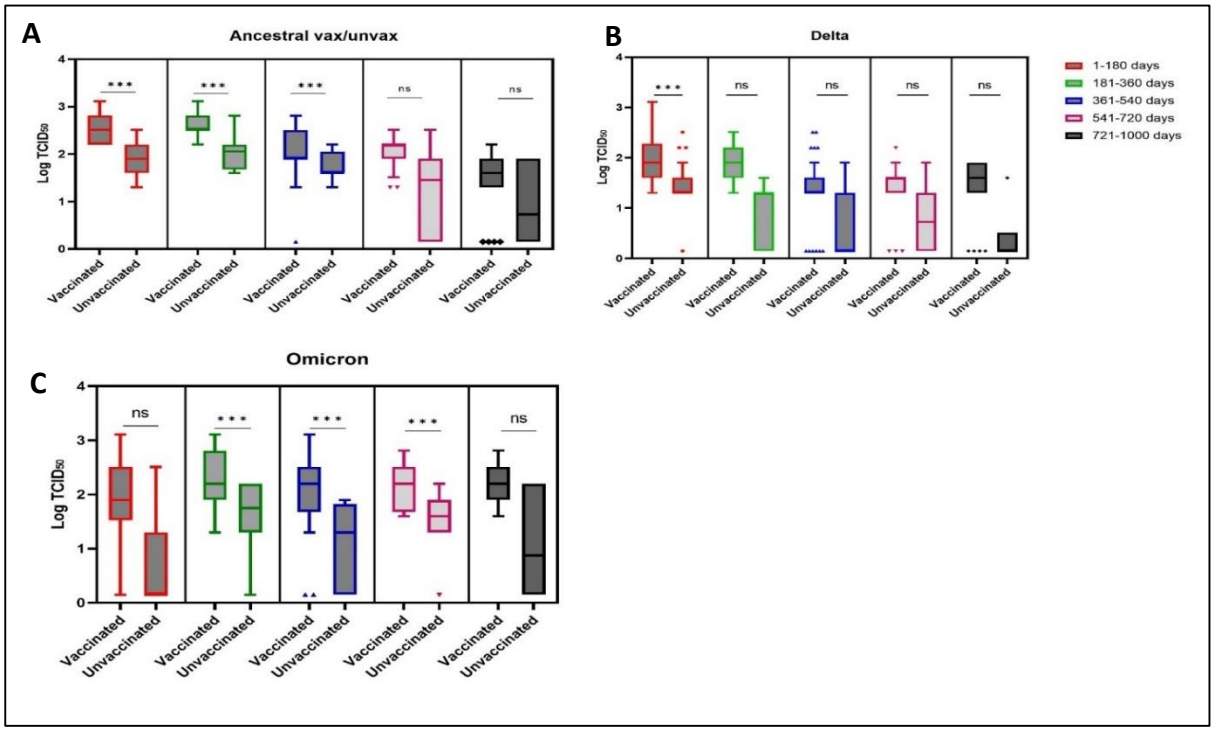

Figure 3.5: Comparison of neutralizing antibody titers between vaccinated and unvaccinated participants over the study period against each variant. A total of 244 samples collected from 100 individuals between 2021 and 2023 at various times post-initial diagnosis. A. Ancestral strain. B. Delta variant. C. Omicron variant. ***P>0.05, ns not significant.

3.3.4. Neutralizing antibody titers and infecting variant

Neutralizing antibody titers against homologous and heterologous strains were compared. Thirty samples collected from 8 to 360 days post symptom onset from people infected with the Ancestral variant, including 20/30 vaccinated, 8/30

unvaccinated, and vaccine history was unknown for 2/30. An additional 36 follow-up samples were collected 540 to 1000 days months post-symptom onset. Neutralizing antibody titers were significantly higher in samples collected 1-180 days (p=0.04) and 181-360 days (p=0.05) post-infection compared to 721-1000 days post-infection (Figure 3.6A). Results suggest that participants maintained their antibody levels against the Ancestral stain, possibly due to receiving booster vaccines before the second bleed and gradually waning over time.

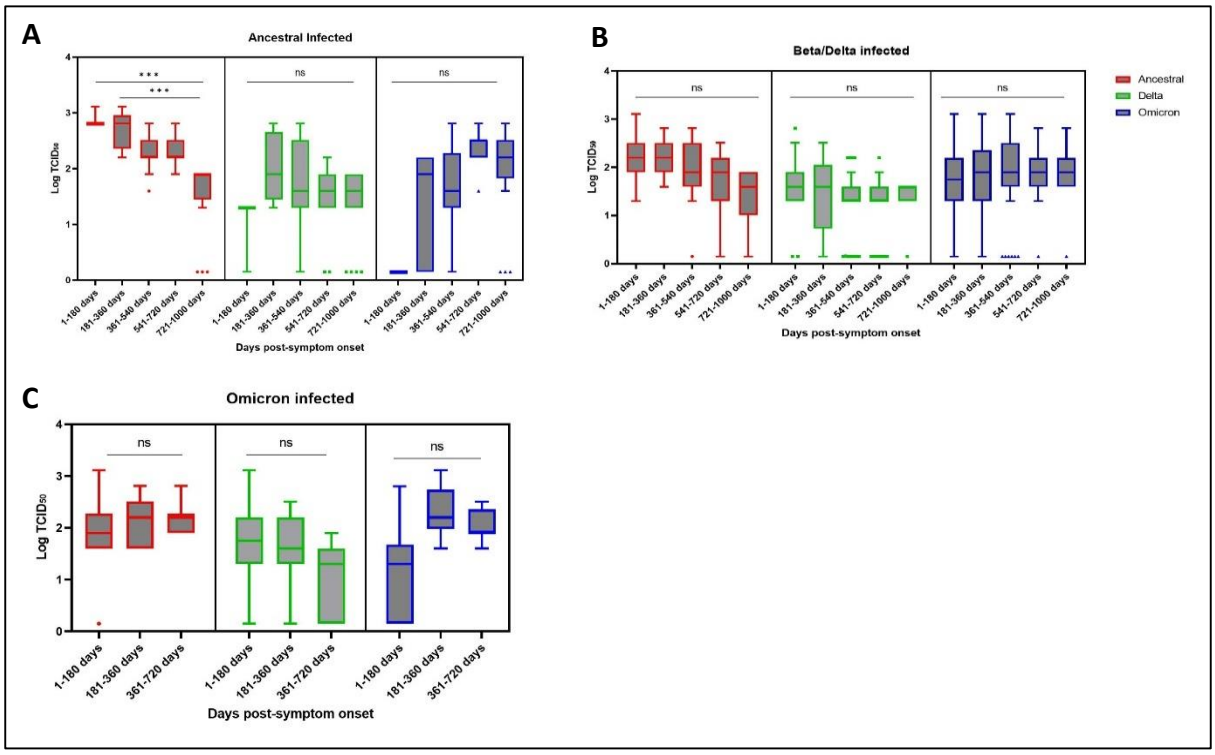

Figure 3.6: Cross-neutralization activity in sera from individuals infected with A. Ancestral (n=66), B. Beta/Delta (n=136), and C. Omicron (n=42). ***P>0.05, ns not significant.

There was no statistically significant difference in neutralizing antibody levels against the Delta variant (p=0.06) (Figure 3.6B) and or against the Omicron variant (p=0.4) (Figure 3.6C), although in both instances there was a decline in levels, a waning of levels. No neutralizing antibody was detected in 6/14 samples against the Omicron variant, possibly because the samples were collected before an endogenous response had developed.

The trends observed against the Ancestral strain and Delta variant suggest that neutralizing antibody levels wane over time but remain detectable. Interestingly, the neutralizing antibody levels against Omicron showed less of a decline. However, as

the Omicron variant is currently in circulation, it is possible that reinfection and/or vaccination is responsible for neutralizing antibody levels.

4. Discussion

The immune correlates of protection are not clearly defined, and hence understanding the kinetics of antibody responses in naturally infected and vaccinated populations may impact on the future role of vaccines. Although the role of neutralizing antibodies in protection against infection and reinfection is not clearly defined, these antibodies are assumed to play some role in minimizing disease severity. Previous studies have shown that neutralizing antibodies remain for at least six months, before starting to wane (Pradenas et al., 2020; Wajnberg et al., 2020). Studies investigating antibody duration after six months have reported waning of neutralizing antibodies 6-9 months post-infection, irrespective of age, sex, or disease severity (Kim et al., 2022). In our study, we extended the duration of testing for samples collected up to 2.5 years post-initial infection and collected 244 samples from 100 individuals with a previous SARS-CoV-2 infection to test for both IgG and neutralizing antibodies against different SARS-CoV-2 variants. Results indicated that IgG antibodies were the strongest early after infection, with the highest antibody levels detected within the first 180 days. However, these gradually decline with time, showing that although there was longevity of antibody, the levels did show a decline. Similar results were observed with the Ancestral strain and the Delta variant regarding neutralizing antibodies indicating a significant decline with time. In contrast, neutralizing antibodies against the Omicron variant seem to increase with time, potentially because the variant was still prevalent at the time of sample collection. Although a small number of individuals reported reinfection, it is also possible that more individuals were asymptomatically infected. This could account for the observed increase in neutralizing titers over time for the Omicron variant. A distinct limitation of the study is the inability to accurately determine if the longevity was enhanced by reinfection, as in many instances, this can be asymptomatic. However, an important outcome was the role of vaccination in antibody titer responses. The population included both vaccinated and unvaccinated people, and a comparison highlighted the benefit of vaccination for maintaining neutralizing antibody titers and, therefore an important role for continuing vaccination programs globally. Results showed that vaccinated individuals had significantly higher IgG and

neutralizing antibodies than the unvaccinated group. Although not statistically significant at 541-720 days and 721-976 days post-onset of symptoms, in most groups, it was still an observable trend that antibody levels were greater in the vaccinated group. Among the 62 individuals from which blood was collected the final year of the study 70.9% (44/62) that still had detectable IgG antibodies were vaccinated, 69.4% (43/62), 64.5% (40/62), and 69.4% (43/62) with detectable neutralizing antibodies against the Ancestral strain, Delta, and Omicron variant were vaccinated against SARS-CoV-2, emphasizing the effectiveness of vaccination in maintaining immunity and showing that additional antigen exposure from vaccination is critical for increasing the level of protection against the different variants. Despite the emergence of VOC and a tendency for lower titers of neutralizing antibodies to be detected against variants, there was detectable cross-neutralization, suggesting that vaccination with the vaccines based on ancestral strain continue to play an important role in inducing cross neutralizing antibodies, even against heterologous variants.

Vaccination has been shown to increase all humoral immunity components, inducing additional plasma cell differentiation from the memory B cell compartment (Wang et al., 2021). In this study, sequential samples were collected between 12 to 976 days post-symptom onset. This allowed for analysis based on different factors, including time of sample collection post-symptom onset, variant of infection, and vaccination history. The findings indicate that anti-S IgG levels and neutralizing titers against the Ancestral strain and Delta variant were high in the first 180 days post-infection, then gradually waned before significantly declining. However, despite the decline, the majority of individuals still at detectable levels of neutralizing antibodies on the final draw. Overall, 85% of individuals retained detectable anti-S IgG antibodies, while 85% and 81% had detectable neutralizing antibodies against the Ancestral strain and Delta variant, respectively. Previous studies show that infection alone provides short-lived protection against reinfection (Cavanaugh et al., 2021) and that vaccination significantly boosts immunity (Wang et al., 2021, Bates et al., 2022).

To summarize, the results indicate that while IgG and neutralizing antibodies wane with time, a significant proportion of individuals still maintained detectable levels even 24 months post-infection. Additionally, the study highlights the considerable variation in neutralizing antibody levels among individuals influenced by different factors. Therefore, conducting longitudinal studies becomes crucial in identifying the immune markers associated with protection, thus enhancing our understanding of SARS-CoV-

2 immunity. This study has other limitations due to the rapid evolution of the outbreak, the emergence of variants, the implementation of vaccine programs, and the resistance of some of the population to receive the vaccine, resulting in a mixed sample cohort. However, the role of vaccination in boosting IgG and neutralizing antibody response was evident, emphasizing the importance of developing further vaccines against emerging variants. Additionally, patient demographics were not recorded at the beginning of the study, including age, comorbidities, the severity of infection, and whether individuals were hospitalized. Previous studies have highlighted the importance of these factors in determining the strength of neutralizing antibodies in individuals. The knowledge from this study can inform public health interventions and optimize vaccine strategies aimed at mitigating the impact of the virus on global health.

Authors Contribution

Litabe M: Conceptualization and design of the study, investigation, methodology, data curation, formal analysis, writing- original draft, reviewing & editing

Bester PA: virus sample acquisition, writing- review and editing

Burt FJ: Conceptualization and design of the study, methodology, supervision, writing–review & editing, funding acquisition

Conflict of Interest:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 5

Dynamics of IgG antibodies against severe acute respiratory syndrome coronavirus 2 in COVID-19 recovered individuals.

Matefo Litabe[1], Felicity Burt[1,2]

1. Division of Virology, Faculty of Health Sciences, University of the Free State, Bloemfontein, South Africa
2. Division of Virology, National Health Laboratory Service, Bloemfontein, South Africa

Abstract

The spike (S) protein of SARS-CoV-2 is found on the surface of the virus and mediates binding to host cells, making it highly immunogenic. Thus, the protein can serve as a useful biomarker in monitoring immunity from natural infection or vaccination and performing seroprevalence studies. The study aimed to determine the duration and dynamics of anti-S IgG antibodies in a cohort of COVID-19-recovered individuals. Three sequential samples were collected from 62 individuals with a previous RT-PCR confirmed SARS-CoV-2 infection between June 2021 and March 2023. Forty-four of the individuals were vaccinated against SARS-CoV-2, while the remaining 19 were unvaccinated. Serum samples were serially diluted four-fold from 1:100 to 1:6400 and tested for anti-S IgG antibodies to determine the endpoint titer for each individual. Antibody titers were significantly higher in samples collected in 2021 compared to 2022 (p=0.001) and 2023 (p=0.0141) and those collected in 2022 compared to 2023 (p=0.0475). Antibody titers were significantly higher in samples from vaccinated individuals than unvaccinated individuals. A positive IgG response was detected in 90% (56/62) of individuals for the three year duration of the study. Overall, 61.29% (38/62) of the individuals showed a decrease in IgG antibodies over time, antibody levels increased in 8.1% (5/62), fluctuating titers were detected in 20.9% (13/62), 6.45% (4/62) had stable antibody titers, and 3.22% (2/62) had no detectable IgG antibodies throughout the study. The results highlight the waning of immunity with time. However although the antibody response may wane, most individuals were able to maintain detectable IgG antibodies for at least two years. The study also showed the importance of vaccination in maintaining higher IgG titers, highlighting the importance of booster vaccinations.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent for coronavirus disease-19 (COVID-19), has spread worldwide with over 769 million confirmed cases and almost 7 million deaths (https://data.who.int/dashboards/covid19/cases?n=c). To date, over 13.4 billion vaccine doses have been administered globally (https://data.who.int/dashboards/covid19/vaccines?n=c). The SARS-CoV-2 genome encodes for four structural proteins, including envelope protein (E), membrane protein (M), nucleocapsid protein (N), and the spike (S) protein. The S protein mediates binding to host cells via interactions with the human receptor angiotensin-converting enzyme 2 (ACE2) (Zou et al., 2020). It is highly immunogenic, making it an excellent target for detecting IgG antibodies (Di Rienzo et al., 2023). The S protein is abundantly present on the surface of the virus, making it easily accessible to the immune system. The S protein is the primary target for neutralizing activity during viral infection, conferring superior protective immunity compared to other viral proteins (Buchholz et al. 2004). Antibodies are usually detectable one week post-symptom onset and provide a suitable target for estimating the proportion of a population exposed to SARS-COV-2 or vaccinated and having an immune response against the virus (Sun et al., 2020). Vaccines, including the BNT16262 by Pfizer, mRN-1273 by Moderna, and Johnson & Johnson's Janssen, are designed to elicit an immune response against the S protein. While detecting IgG antibodies is not ideal for diagnosing acute infections, they allow the study of humoral immune responses to SARS-CoV-2 and allow screening of SARS-CoV-2 antibodies in the population, which facilitates seroprevalence estimations and readily assesses the persistence of a detectable immune response in the population or at the individual level which can all improve vaccine strategies. The immune correlates of protection are not clearly defined. However, studies show that both the humoral and cellular responses play a role in the protection and clearance of the virus (Feng et al., 2021). The detection of IgG antibodies using serological assays is a useful biomarker for monitoring natural exposure or vaccination. Previous studies have shown waning of IgG antibodies post-infection with SARS-CoV-2 over time (Hernandez-suarez and Murillo-zamora, 2022), raising concern regarding long-term protection against reinfection and the possibility of attaining herd immunity. Some studies have reported a decline within the first three

months post-onset of symptoms, while others show persistence for up to one year (Duysburgh et al., 2021; Haveri et al., 2021; Marot et al., 2021). The duration of detectable IgG antibody responses has been shown to vary, which may be due to multiple factors, including the population, infecting variant, and method of detection. Studies conducted in Austria demonstrated the persistence of antibodies one-year post-infection (Nunhofer et al., 2022), whereas other studies have reported contrasting results, with a rapid decline in detectable IgG within four to five months post-infection or vaccination (Lagousi et al., 2022; Abou-Saleh et al., 2022). This shows that more is yet to be understood regarding the long-term persistence and role of the humoral immune response against SARS-CoV-2. Other studies also show that antibody persistence reduces the risk of SARS-CoV-2 reinfection (Hansen et al. 2021). These observations make it important to attempt to understand the long-term persistence of SARS-CoV-2 antibodies and the dynamics and changes over time. This study aimed to determine the duration and dynamics of IgG antibodies in COVID-19-recovered individuals.

2. Methods and Materials

2.1. Ethical approval

The study was approved by the University of the Free State Health Sciences Research Ethics Committee (UFS-HSD2020/2001/2601-0005) and the University of the Free State Environment and Biosafety Ethics Committee (UFS-ESD2020/0181/22). Written informed consent was obtained from each participant before the collection of blood.

2.2. Serum samples

A total of 62 participants with reverse-transcriptase polymerase chain reaction (RT-PCR)-confirmed SARS-CoV-2 infections were enrolled in the study, and initial samples were collected between June 2021 and December 2021. Follow-up samples were collected between June- September 2022 and January- March 2023. Samples were diluted four-fold from 1:100 to 1:6400 to determine the endpoint of the IgG antibody titers using an in-house anti-S IgG ELISA.

2.3. Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was an in-house assay using recombinant S protein expressed in human embryonic kidney (HEK-293) cells and

validated previously (Matefo et al. 2022). Unless stated otherwise, all volumes were 100µL/well, plates were incubated for one hour at 37°C and washed for 3 x 15 seconds with 0.1% Tween 20 in phosphate-buffered saline (PBS) pH 7.0. Briefly, 96-well PolySorp microtitre plates (Nalge Nunc International Corporation, USA) were coated with 100µL of 3.5µg/mL of purified recombinant SARS-CoV-2 S protein diluted in carbonate buffer pH 8.6 overnight at 4°C. Control wells contained carbonate buffer without recombinant antigen. Plates were washed and blocked with 200µL/well 10% skimmed milk/PBS. Serum samples were diluted fourfold from 1:100 to 1:6400 in 2% skimmed milk/PBS and added to antigen-coated wells and control wells. Duplicate positive (C++) and negative (C-) controls were included on each plate. Positive reactors were detected using anti-human IgG horseradish peroxidase (HRPO) conjugate diluted 1:2000 (SeraCare Life Sciences Inc., Milford, USA) and 2,2'-azino di-ethyl-benzothiazoline-sulfonic acid peroxidase substrate (ABTS) (SeraCare Life Sciences Inc., Milford, USA). Plates were incubated for 10 minutes at room temperature (22-24°C) in the dark, and the optical density (OD) values were read at 405 nm. Net OD values were determined for each sample as follows: net OD = OD in wells with recombinant SARS-CoV-2 antigen minus OD in wells with no antigen.

OD values were normalized to account for variability by determining the percentage of positive values based on positive control serum (Paweska et al., 2007) as follows: (net OD of test sample/net OD of C++) × 100. A cutoff value of 30%, which differentiated positive samples from negative samples, was determined in the previous validation (Matefo et al., 2022, Chapter 3).

2.4. Statistical analysis

Statistical analysis and graphical presentation of results were performed using GraphPad Prism version 9.2.0. Differences in antibody titers between participants were determined using the analysis of variance (ANOVA) test, and a p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Reactivity of convalescent COVID-19 sera against SARS-CoV-2 S antigen

A total of 62 individuals previously infected with SARS-COV-2 were recruited for the study in 2021. Blood was collected on three occasions for each individual, between March 2021 and March 2023, for IgG testing resulting in 186 serum samples. A total

of 46/62 of the individuals were vaccinated against SARS-CoV-2, and 16/62 were unvaccinated. Initial samples collected in 2021 were collected between March and December. Follow-up samples were collected between June and August 2022 and between January and March 2023. Seven individuals reported reinfection, six in 2022 and one in 2023. Samples were collected 12 and 976 days post-symptom onset, and grouped according to the year of diagnosis.

Serum samples were diluted fourfold from 1:100 to 1:6400, and IgG endpoint titers were determined using ELISA. The OD values were converted to percentage positive (PP) values to account for any variability between runs, and the cutoff value of 30% was used to differentiate between positive and negative samples. The endpoint titer was expressed as the log of the highest dilution with a PP value above the cutoff.

A total of 186 samples were collected 12 to 976 days post-symptom onset, and grouped according to the year after initial diagnosis. A total of 68 serum samples were collected within the first year post-symptom onset (Figure 3.1 A). Among these, 49 individuals were vaccinated, while the remaining 19 were unvaccinated. Antibodies were detected in 66/68 samples, and 2/68 from the unvaccinated group had PP values falling below 30%. One of the negative samples was collected 13 days post-symptom onset, while the other was collected 33 days post-symptom onset. IgG antibody titers were significantly higher in the vaccinated group compared to the unvaccinated group for the 2021 samples (p=0.001) (Figure 3.2). Eighteen patients had IgG titer of ≥1:6400, 16/68 had IgG titer of 1:1600, 15/68 had IgG titer of 1:400, and 18/68 had IgG titer of 1:100.

A total of 96 samples were collected between 366 to 731 days post-symptom onset, antibody titers are shown in year 2 (Figure 3.1B). This group included 75 samples from vaccinated individuals and 21 from the unvaccinated cohort. Among the vaccinated patients, 69/75 had detectable IgG antibodies, while six patients had values below the cutoff of the assay. IgG antibodies were detectable in 16/21 of unvaccinated patients, with 5/21 having antibody levels below the cutoff of the assay. The IgG titers were significantly higher in the vaccinated group than the unvaccinated group (p=0.0161) (Figure 3.2). Notably, the titer of IgG in the second-year post-symptom onset was significantly lower (p=0.0141) compared to the first year, suggesting that the antibody levels were waning with time despite possible boosting from vaccination. Among these samples, 12/96 had IgG a titer ≥1:6400, 22/96 had IgG titer of 1:1600, 30/96 had IgG a titer of 1:400, and 26/68 had IgG titer of 1:100.

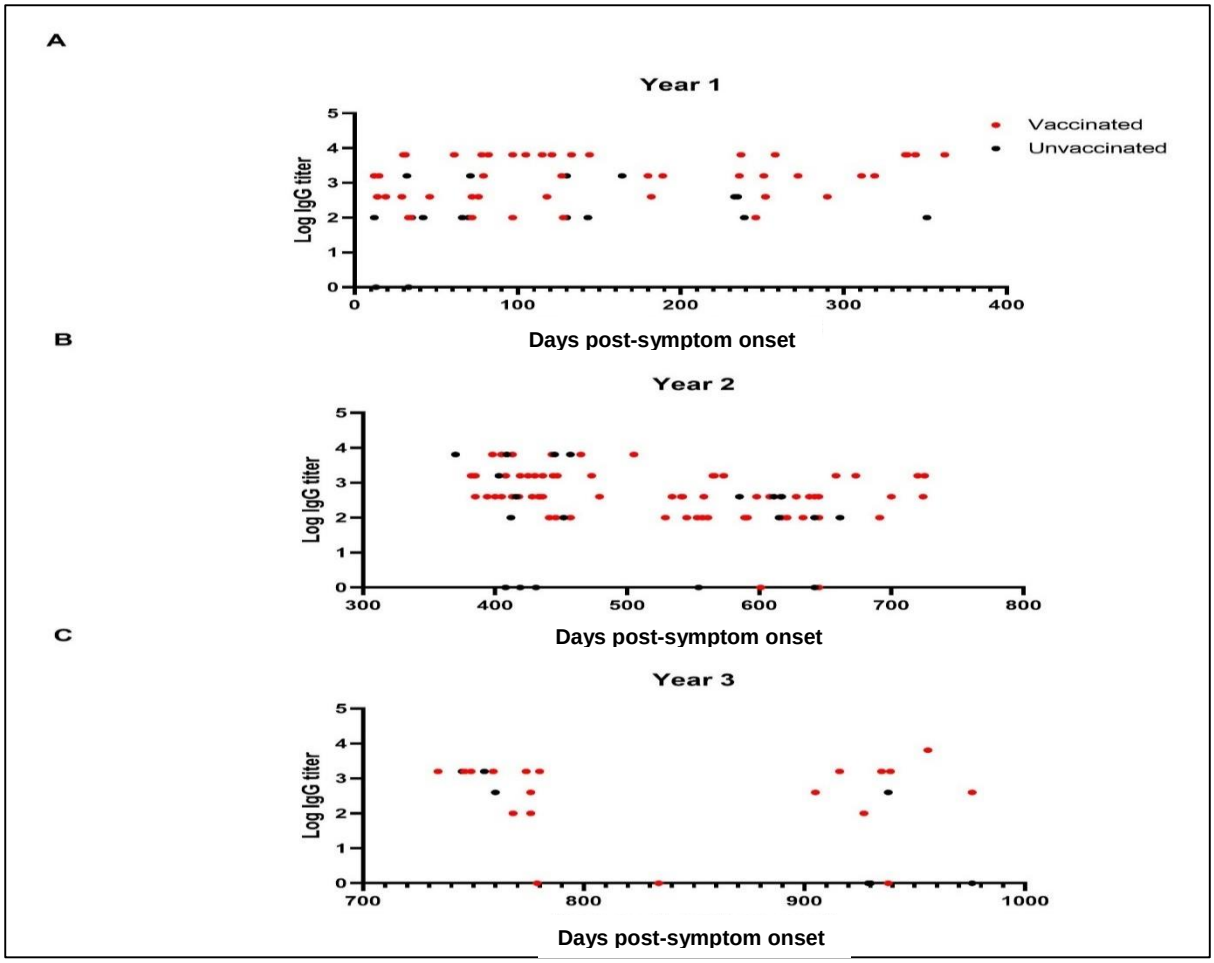

Figure 3.1 Antibody titers against SARS-CoV-2 over a three-year period. Serum samples were diluted fourfold from 1:100 to 1:6400 to determine the endpoint titer. Results are presented as the log of the reciprocal of the highest dilution that was positive. Samples were grouped based on the time of sample collection post-symptom onset. A. Samples collected between 1-365 days post-symptom onset. B.Samples collected between 366-731 days post-symptom onset. C. Samples collected between 732-1000 days post-symptom onset.

During year 3, 28 samples were available for analysis collected between 732 and 1000 days post-symptom onset (Figure 3.1C). The group included 21 samples from vaccine recipients and seven unvaccinated. Anti-S IgG antibodies were detected in 22/28, and 6/28 patients tested negative. There was no statistically significant difference in IgG titers between vaccinated and unvaccinated (p=0.1834) (Figure 3.2C). One individual had an IgG titer of ≥1:6400, 11/28 had an IgG titer of 1:1600, 5/28 had an IgG titer of 1:400, and 5/28 had an IgG titer of 1:100. (Figure 3.3) IgG titers were significantly higher in samples collected in the first year compared to those collected in the second year (p= 0.0163) and third year (p=0.00141). IgG titers were also significantly higher

in samples collected in year 2 (0.0475) compared to year 3. The findings suggest that while most individuals maintain detectable levels of anti-S IgG antibodies for an extended period after infection, there appears to be a decline in the antibody titer, which might indicate a waning of immunity to reinfection. The variation in immune response between vaccinated and unvaccinated individuals shows the potential benefits of vaccination against SARS-CoV-2.

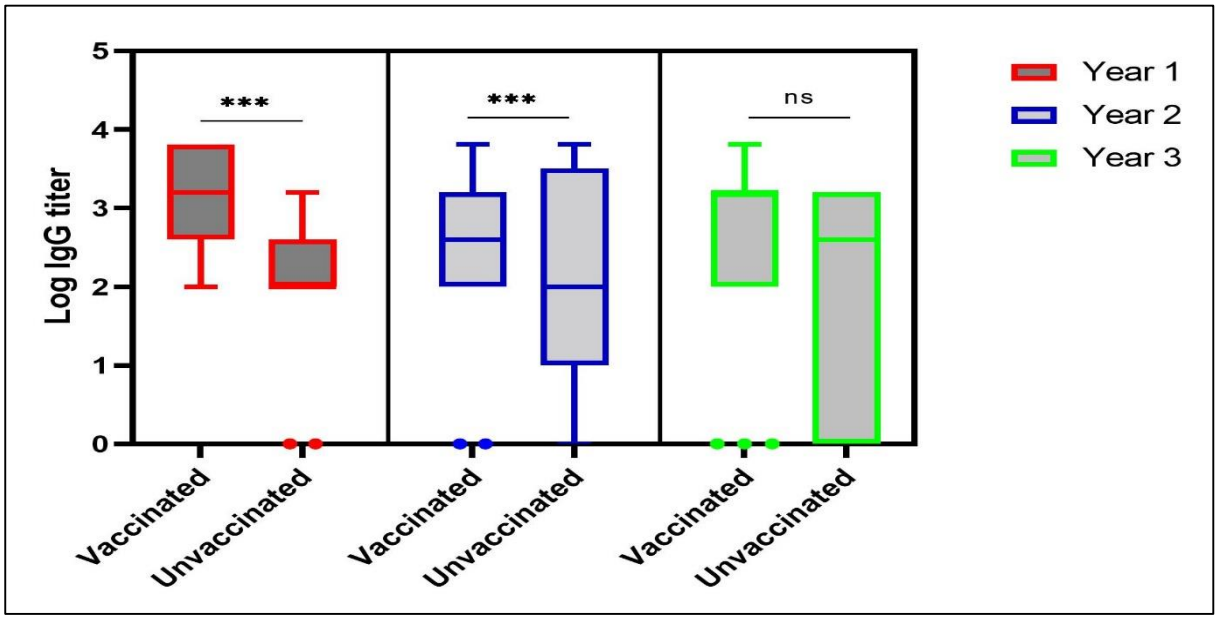

Figure 3.2: Comparison of IgG titers between vaccinated and unvaccinated patients over time post-symptom onset. ***P>0.05, ns not significant.

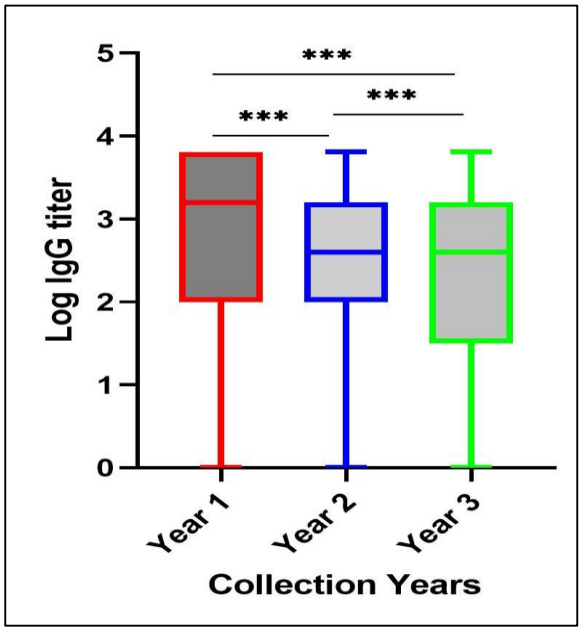

Figure 3.3: Comparison of IgG titers for years 1 to 3. Sequential samples were collected from 62 patients on three occasions, and endpoint IgG titer was determined. ***P>0.05.

3.2 Trajectory of antibody dynamics between patients

Variations in antibody kinetics over time were observed between different individuals; thus, samples collected from individuals were grouped depending on the trajectory they were following to further investigate the duration and kinetic trends of antibody responses. Patients were characterized based on increasing, decreasing, fluctuating, or stable IgG titers during the three-year period after the initial illness. Decreasing IgG levels were defined as a reduction in antibody levels by at least one log from one collection period to the next. Increasing IgG levels was defined as a rise in IgG levels by at least one log between collection periods. Patients in which IgG levels remained the same throughout the study period were considered stable. Fluctuating IgG levels were defined as a variation in antibody levels between collection periods, with IgG levels increasing or decreasing. Table 3.1 summarizes the total number of patients in each group and their vaccination history, and IgG antibody titer trajectories are shown in Figure 3.3A-D. Most individuals exhibited a decrease in IgG antibody titers over the three-year period (Figure 3.4A). A decline in titers was noted in 38/62 (61.3%), including 7/39 (three vaccinated and four unvaccinated) patients in which antibody titers declined to undetectable levels. Antibody titers increased in five (8.1%) individuals over the collection period, including three vaccinees and two unvaccinated (Figure 3.4B), possibly due to vaccine boosting the immune response or reinfection. Four (6.5%) individuals maintained stable IgG titers over the three-year period (Figure 3.4C), which also may be due to vaccine or reinfections. Fluctuating antibody titers were recorded for 13 patients, including two unvaccinated and 11 vaccinated. Although it is an assumption, receiving vaccine or having a reinfection is likely due to boost the responses. Two patients had antibody titers below the detection limit of the assay throughout the study. Six individuals who reported reinfection showed a decline in IgG titers, with a fourfold decrease between collections but maintaining a detectable titer of 1:100. One individual who reported reinfection had fluctuating IgG titer, 1:400 on the first, 1:1600 on the second, and 1:100 on the final collection. The individual reported reinfection before the second collection, which may explain the increase in IgG titer.

Table 3.1: Antibody trajectories in COVID-19 previously infected individuals

Antibody Trajectory	Total patients	
Decline	38	Vaccinated n=30 Unvaccinated n=8
Increase	5	Vaccinated n=3 Unvaccinated n=2
Stable	4	Vaccinated n=2 Unvaccinated n=2
Fluctuating	13	Vaccinated n=11 Unvaccinated n=2
Undetected	2	Unvaccinated n=2

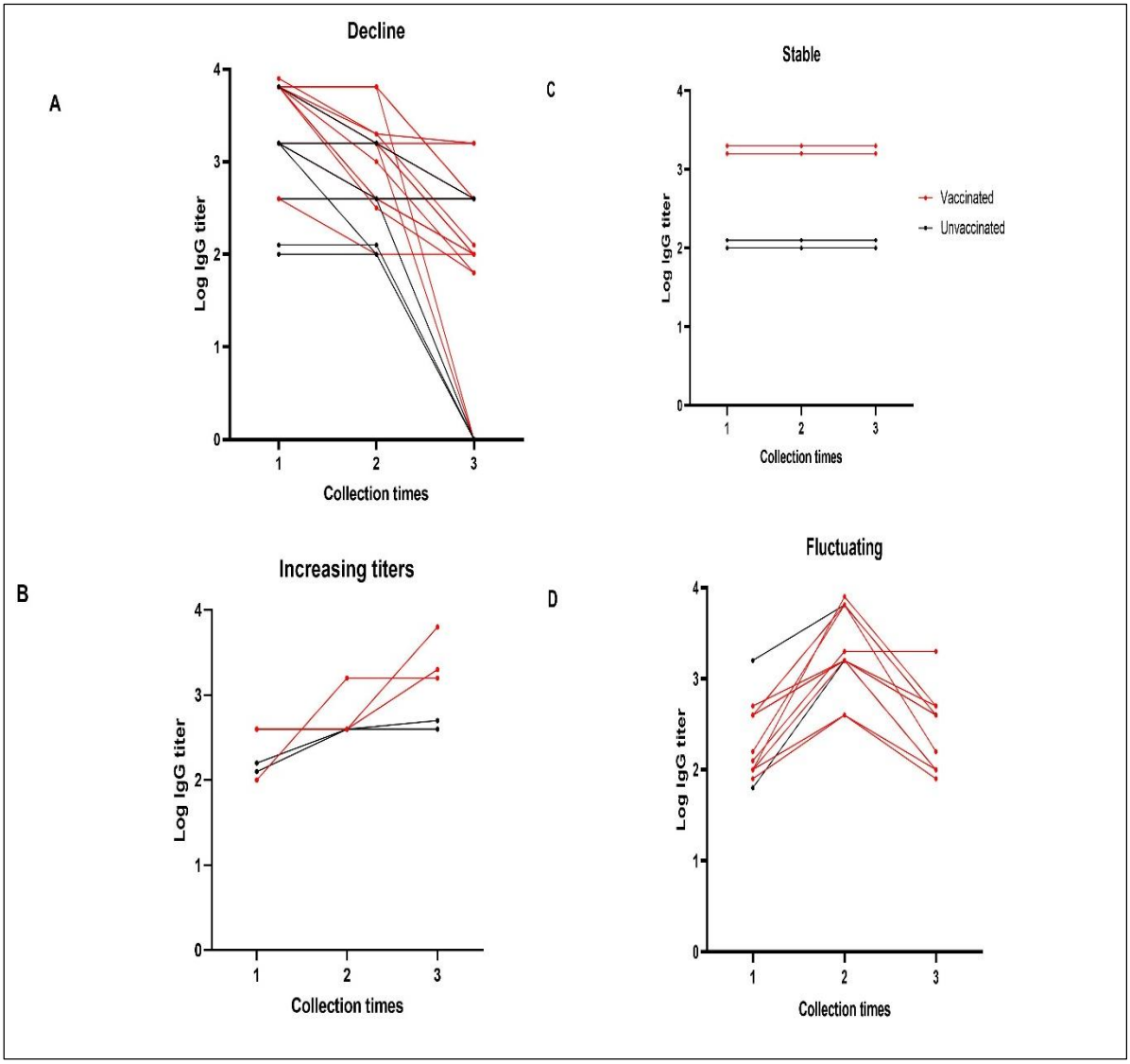

Figure 3.4: Trajectories of IgG antibody responses in individual patients over three years. A. Decreasing IgG titers. B. Increasing IgG titers. C. Stable IgG titers. D. Fluctuating IgG titers. Red lines represent vaccinated individuals and black line unvaccinated group.

4. Discussion

Studying the dynamics of antibodies is important for determining the chances of subsequent reinfection and understanding the overall evolution of COVID-19, especially due to its mutating nature, with new variants constantly emerging. Most studies have focused on the duration of antibody levels over a short period (Lea et al., 2022; Yousefi et al., 2022). This study investigated the dynamics and duration of IgG antibodies in a diverse group of previously infected SARS-CoV-2 individuals between March 2021 and March 2023, with and without vaccination over three years. SARS-CoV-2 anti-S IgG seropositivity was sustained in 90.3% (56/62) of the patients for three years post-symptom onset; 61.3% of the patients showed a decrease in IgG titer, 8.1% showed an increase, 20.9% had fluctuating titers, 6.5% maintained a stable IgG titer, and 3.2% had an undetectable IgG response. Only 11.3% (7/62) of individuals reported reinfection in the study. Although more may have potentially been reinfected with asymptomatic SARS-CoV-2 and not tested, the number of reinfections suggests that the persistence of antibodies may have reduced the chances of reinfection. Other studies have found that the presence of antibodies reduces reinfection risk (Hansen et al., 2021; Marsden et al., 2021). This also shows that infection does not provide total immunity. Although most patients show a decline in IgG titer, vaccinated patients had significantly higher IgG antibody titers compared to the unvaccinated, suggesting that vaccination may be important in maintaining higher antibody responses. Results from one study showed that even though there was a slight decline in antibodies eight months post-infection in previously infected individuals, anti-S memory B cells increased during this period (Dan et al. 2021). Another study showed that anti-S antibodies remained relatively stable for up to 12 months post-infection, and memory B cells were retained in repertoires for at least one year post-infection, with all components of the humoral immune response increasing after vaccination (Wang et al. 2021). The fact that most (61.3%) of individuals showed a decline in IgG titers, with only 11.29% reporting reinfection, also shows that other parts of the immune system are playing a role in protection. The immune correlates of protection are not clearly defined, and it is likely that both cellular and humoral responses play a role. Antibody responses are easy to measure and, hence, have been used as a biomarker of immune responses and duration of immunity. However, this does have limitations, as cellular responses likely play a role. One study found that T-cell immune memory could

be detected in a cohort of patients, including those that had lost detectable neutralizing antibodies one year post-infection, suggesting that T cells retained the ability to mediate cellular immunity in patients who had lost detectable neutralizing antibody responses (Guo et al., 2022). Another study showed that CD4+ T-cells can be maintained at high levels for at least eight months post-infection (Dan et al. 2021).

While IgG waning and reinfection within a year have been reported for seasonal coronaviruses, IgG against SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) remained detectable 1-3 years post-infection (Cao et al., 2010; Payne et al., 2016) and even up to 6.9 years regardless of disease severity (Alhabbab et al., 2022). Current studies have determined the duration of SARS-CoV-2 for up to 18 months post-symptom onset (Vanshylla et al., 2021; Chivu-Economescu et al., 2022; Yousefi et al., 2022). In our study, IgG antibodies were detected in 90.3% of participants 2-3 years post-infection, suggesting longevity of antibodies, which may have been boosted by reinfection and/or vaccination. Additionally, these results show that the immune system can maintain long-lived immune memory, potentially contributing to faster and more effective immune responses upon re-exposure to SARS-CoV-2. However, these results, like previous studies, show that even if herd immunity is reached, it will not eliminate SARS-CoV-2. The virus will continue circulating, and waning antibodies will likely increase the chances of reinfection. Although vaccination cannot provide long-life protection, it can play a massive role in boosting immunity and reducing the likeliness of reinfection. This shows the importance of continued vaccination. It would have been interesting to correlate potential variations in the disease severity among these patients. Immune responses to SARS-CoV-2 vary widely among individuals, other studies also suggest that the severity of the disease, age, sex, and comorbidities may play a role in influencing SARS-CoV-2 antibody responses (Vanshylla et al., 2021; Petersen et al., 2022). The limitation of the study was that it did not capture all the relevant variables that could affect the dynamics of IgG, including age, gender, severity of disease, and patients pre-existing conditions. Additionally, the study had a small number of patients and we were unable to determine if there were undiagnosed mild or asymptomatic reinfections. The main strength of the study is that it spans a substantial time frame, which, according to our knowledge, has previously not been done, giving a comprehensive understanding of how IgG titers may change over time. Additionally,

the study included a diverse group of participants, both vaccinated and unvaccinated. Thus, the findings provide insights into IgG titers among different individuals. These findings show that detection of anti-S IgG remains a useful biomarker for surveillance of natural infections and/or vaccine. Studying antibodies induced only by natural infection, their protective nature, and how they change with time is also important. The N protein could be used as a target for natural infections only as a majority of SARS-CoV-2 vaccines primarily target the S protein.

In summary, the results show the complexity of immune responses and the variability in how individuals develop and maintain immunity. A small number of individuals were able to naturally maintain higher IgG titers over time, while the majority resulted in a decline in IgG levels with time, suggesting that most individuals might benefit from booster vaccinations to ensure sustained antibody titers. These results provide insights into the dynamics of SARS-CoV-2 immune response over a period of three years.

Authors Contribution

MM Litabe: Investigation, methodology, data curation, formal analysis, writing- original draft, reviewing and editing

FJ Burt: Methodology, supervision, writing– review and editing, and funding acquisition

Conflict of Interest:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 6

In-vitro Assessment of Phela and Components of Phela in Modulating the Innate Immune Response against SARS-CoV-2

MM Litabe[1], PA Bester[1,2], M Matshabisa[3], FJ Burt[1,2]

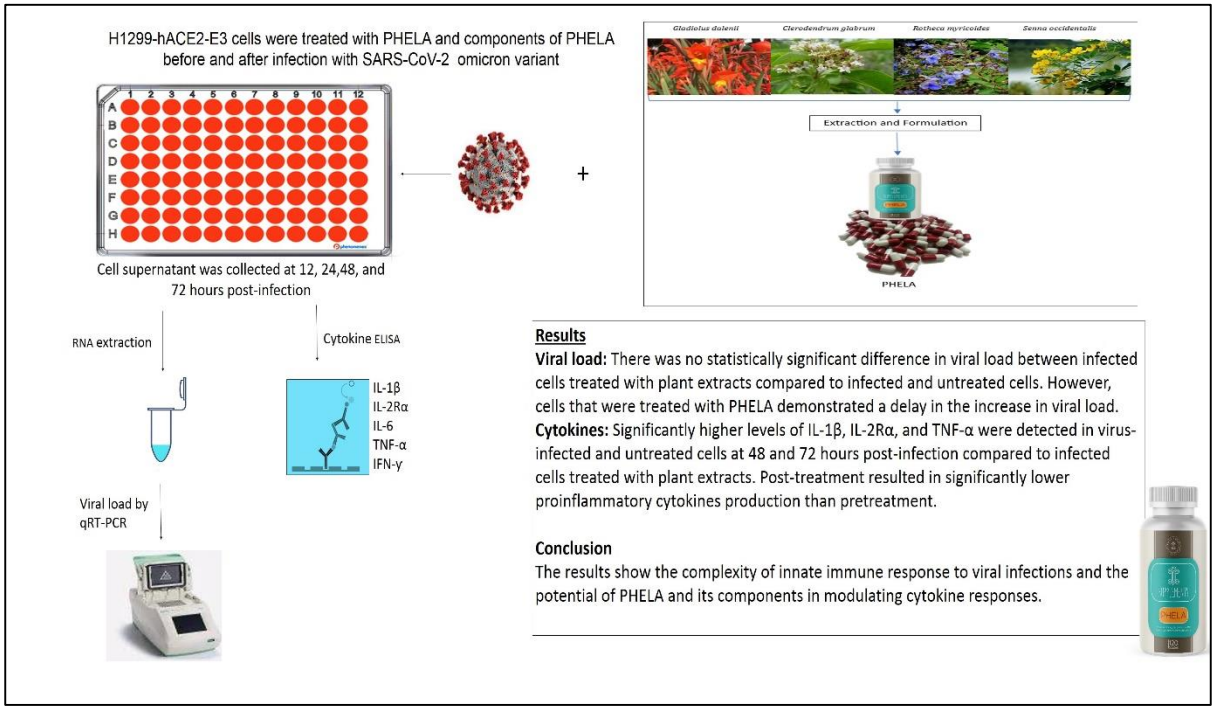
1. Division of Virology, Faculty of Health Sciences, University of the Free State, Bloemfontein, South Africa
2. Division of Virology, National Health Laboratory Service, Bloemfontein, South Africa
3. Department of Pharmacology, Faculty of Health Sciences, University of the Free State, Bloemfontein, South Africa

Abstract

Severe acute respiratory syndrome coronavirus (SARS-CoV)-2 was responsible for severe disease, which triggered an uncontrolled release of proinflammatory cytokines in some patients, resulting in a cytokine storm associated with disease severity. Traditional medicinal plants have been proposed as promising, cost-effective treatments for SARS-CoV-2, with studies showing the potential to induce protection against different viral infections. Phela is a traditional medicine prepared from the extracts of four South African plants and has been proposed for use as an immune booster. This study investigated the potential of Phela and the individual components to modulate the release of cytokines in SARS-CoV-2 Omicron-infected mammalian cells and to investigate the influence of the plant extracts on viral replication. Cells were treated with the plant extracts before or after infection with SARS-CoV-2. Subsequently, cell culture media was collected at 12, 24, 48, and 72 hours post-infection and tested for levels of interleukin (IL)-1β, IL-2Rα, IL-6, IL-10, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ cytokines. RNA was extracted from the media to measure viral load. There was no statistically significant difference in viral load between infected cells treated with plant extracts compared to infected and untreated cells. However, cells that were treated with Phela demonstrated a delay in the increase in viral load. Significantly higher levels of IL-1β, IL-2Rα, and TNF-α were detected in virus-infected and untreated cells at 48 and 72 hours post-infection compared to infected cells treated with plant extracts. Post-treatment resulted in significantly lower proinflammatory cytokines production than pretreatment. The

results show the complexity of innate immune response to viral infections and the potential of Phela and components of Phela in modulating cytokine responses.

Graphical Abstract



1. Introduction

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in December 2019 in Wuhan, China, resulting in a pandemic. Infection with SARS-CoV-2 can result in dysregulated release of proinflammatory cytokines, resulting in a cytokine storm. In particular, IL-6 has been identified as one of the main proinflammatory cytokines and used as a disease severity biomarker. High levels of IL-6 have been associated with disease severity, organ failure, and death (Lavillegrand et al., 2021). Although vaccines have been developed against SARS-CoV-2, effective and safe antivirals are still needed to treat and manage the disease. Currently, some attempts at treatment have focused on repurposing drugs, including human immunodeficiency virus (HIV) antivirals such as HIV-1 protease inhibitors lopinavir (Mahdi et al., 2020), the hepatitis C virus protease inhibitor danoprevir (Chen et al., 2020), the influenza antiviral favipiravir (Kaptein et al. 2020), and the RNA polymerase inhibitor, remdesivir (Pruijssers et al., 2020; Vangeel et al., 2022). The strategy of repurposing provides a potentially rapid trajectory toward an approved treatment. Traditional medicines have been proposed as affordable

treatments and alternatives for patients suffering from COVID-19. At the beginning of the COVID-19 pandemic, traditional medicines from plant extracts were used in different countries as a form of treatment (Nie et al., 2021; Tang et al., 2021; Flórez-álvarez et al., 2022). Previous studies have shown the effectiveness of traditional medicinal plants against various viral infections (Mehrbod et al., 2018; Mehrbod et al., 2019). Traditional medicines are proposed to strengthen healing and regeneration processes by modulating the host's immune responses (Xu et al., 2023). Traditional medicines can potentially interfere with various steps of the viral replication cycle and induce anti-inflammatory responses that may play a role in reducing severity of illness (Chambon et al., 2023). Phela is prepared from four South African traditional medicinal plant extracts, combining *Gladiolus dalenii*, *Senna occidentalis, Rotheca myriciodes,* and *Clerodendrum glabrum* at specific ratios. Using a rodent animal model, Phela was shown to have immunostimulatory effects and restored cyclosporine-induced immunosuppression, indicating activation of IL-2. (Lekhooa et al., 2012). In this study, we tested Phela and its four components, a traditional medicinal plant, *in vitro* to determine its immunomodulatory effects against SARS-CoV-2 infected cells. Phela is a traditional herbal medicinal plant under development as an immune booster in South Africa. This study aimed to investigate the potential of plant extracts to modulate cytokine responses in SARS-CoV-2 Omicron-infected mammalian cells by investigating viral RNA load and cytokine levels related to cytokine storms in cells infected with the virus.

2. Methods and Materials

2.1. Ethics consideration

The study was approved by the University of the Free State Health Sciences Research Ethics Committee (UFS-HSD2020/2001/2601-0005) and the University of the Free State Environment and Biosafety Ethics Committee (UFS-ESD2020/0181/22).

2.2. Cells, Plant Materials, and Virus

H1299-hACE2-E3 cells, a human lung cell line overexpressing human angiotensin converting enzyme (ACE) 2 receptor (kindly donated by Professor Alex Sigal, African Health Research Institute, University of KwaZulu-Natal) infected with SARS-CoV-2 Omicron were used to determine the cytotoxicity and antiviral activity, or immune modulatory activity of the different plant extracts. Cells were grown in Roswell Park

Memorial Institute (RPMI) 1640 media supplemented with 10% fetal bovine serum (FBS), penicillin (100IU/mL)**,** and streptomycin (100mg/mL) in a 5% CO_2 atmosphere at 37ºC. Growth media for the cytotoxicity assays contained 2% FBS and no additional supplements.

The plant extracts, including Phela, *Gladiolus dalenii*, *Senna occidentalis, Rotheca myriciodes,* and *Clerodendrum glabrum,* were kindly provided by Professor Motlalepula G. Matsabisa from the Department of Pharmacology, University of the Free State, Bloemfontein, South Africa. Phela is manufactured by the Indigenous Knowledge Systems Lead Programme, Department of Pharmacology, Faculty of Health Sciences, University of the Free State. Extracts were obtained from four plants using hydro-alcoholic extraction through maceration, dried into a homogenous powder, and then sterilized by gamma irradiation. The powder is tested before and after irradiation for bacterial and fungal contamination. To produce Phela, the plant extracts were combined at specific ratios and supplied as a powder. Stock solutions of the plant extracts were prepared in deionized water at a final concentration of 2mg/mL. Stock solutions were filter sterilized and stored in aliquots at -80 before use. SARS-CoV-2 Omicron variant was isolated from a human sample using h1299-hACE2-E3 cells and viral stocks stored at -80ºC prior to use, as described in Chapter 4, section 2.4. The tissue culture infectious dose 50 ($TCID_{50}$) was determined by performing tenfold serial dilutions of virus stock on h1299-hACE2-E3 cells in flat-bottomed 96-well plates, from 10^{1} to 10^{-7}, in replicates of six and calculations done using the Reed–Muench method (Reed & Muench, 1938; Ramakrishnan, 2016). All infectious studies were performed in a biosafety level 3 laboratory.

2.3. Cell cytotoxicity assay

The cytotoxicity of the plant extracts was determined using a CellTiter Aqueous One Solution Cell Proliferation Assay (MTS) [3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]-based viability assay (Promega, Wisconsin, USA), to determine a suitable dose for treating h1299-hACE2-E3 cells in which the plant extracts were not toxic. To test for cell viability, a monolayer of $1x10^{5}$ H1299-hACE2-E3 cells was seeded into a 96-well plate and treated with plant extracts diluted twofold from 1600µg/mL to 100µg/mL, then 80µg/mL to 10µg/mL and incubated at 37ºC. Seventy-two hours post-treatment, the cell culture supernatant was removed, the cells were treated with cell proliferation solution, and the cell viability was

measured according to the manufacturer's instructions. Briefly, 20µL of MTS reagent was added directly to the wells, and cell plates were incubated at 37ºC for 2 hours. Absorbance was measured at 490nm on a SpectraMax Plus384 reader (Molecular Devices; Sunnyvale, CA). The average of the duplicate experiments of each sample was obtained. Corrected absorbance was determined by subtracting the absorbance value obtained from culture media only from that obtained in cells treated with plant extracts, and the percentage of viable cells was calculated using the equation below. Dose-response curves were constructed, and the concentration causing death in 50% of the cells (CC_{50}) was determined.

% cell viability= ((sample-media)/(control-media)) x 100

2.4. Assessment of the effect of the plant on viral load

2.4.1. Pretreatment of virus with immune modulator

H1299-hACE-E3 cells were first treated with 80µg/mL of each plant extract, including Phela, at 37ºC for 30 minutes in a humidified 5% CO_2 incubator. Post-incubation, 100µL of $1x10^5$ h1299-h1299-ACE2-E3 cells were infected with 50µL 100 $TCID_{50}$ treated SARS-CoV-2 virus in a final volume of 200µL/well and incubated in a humidified 5% CO_2 incubator at 37ºC. A total of 200µL/well of cell supernatant was collected from each well at 12, 24, 48, and 72 hours post-infection to determine the viral load and selected cytokines using ELISA, separate wells used for each time point. A total of 28 wells were used for each plant extract, 24 used for cytokine studies and 4 for viral replication experiments.The viral load was determined at each time point from one sample. Each cytokine level was tested at each time point using two biological replicates. Virus-infected cells, not treated with plant extracts, and untreated and uninfected were used as controls. To normalize the data obtained, all cell supernatant was stored at -20ºC before use. Each sample had a single freeze-thaw, using the same control, and all were tested on the same day.

2.4.2. Treatment of cells with plant extracts post-infection with SARS-CoV-2

A total of 100µL of $1x10^5$ h1299-hACE2-E3 cells seeded in a 96-well plate were infected with 50µL 100 TCID50 of SARS-CoV-2 Omicron virus and incubated for 30 minutes at 37ºC to allow the adsorption of the virus. Post-infection cells were treated

with 50µL of 80µg/mL of each plant extract and Phela and incubated in a humidified 5% CO_2 incubator at 37ºC at a final volume of 200µL/well. The viral load was determined at each time point from one sample. Each cytokine level was tested at each time point using two biological replicates. A total volume of 200µL of cell culture supernatant was collected from each well at 12, 24, 48, and 72 hours post-infection to determine viral load and cytokine secretion, separate wells used for each time point. Virus-infected cells, not treated with plant extracts, and untreated and uninfected were used as controls. To normalize the data obtained, all cell supernatant was stored at -20ºC before use. Each sample had a single freeze-thaw, used the same control, and all were tested on the same day.

2.4.3. Viral RNA Extraction

RNA was extracted from the cell culture supernatant of infected cells using the Zymo Quick RNA Viral extraction kit (Zymo Research, USA) according to the manufacturer's instructions. All centrifugation steps were performed at 16 000 x *g*. Briefly, 200µL of cell culture supernatant was added to 200µL RNA shield and mixed with 800µL viral RNA buffer. The mixture was transferred to a Zymo-SpinTM IC column in a collection tube and centrifuged for 2 minutes. A volume of 500µL of viral wash buffer was added to the column and centrifuged for 30 seconds. The RNA was washed with 500µL of 96% ethanol and centrifuged for 1 minute. RNA was eluted by adding 15µL of RNase-free water to the column matrix and centrifuging for 30 seconds. Eluted RNA was stored at -80ºC before use.

2.4.4. Real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

The real-time qPCR was performed using the Allplex 2019-nCoV assay (Seegene, Seoul, South Korea). The assay is designed to detect the RNA-dependent RNA polymerase (RdRP) and the nucleocapsid (N) genes specific to SARS-CoV-2 and the envelope (E) gene for all Sarbecovirus, including SARS-CoV-2. Briefly, the master mix was prepared by adding 5µL of 2019-nCoV MOM, 5µL of buffer 5×, 5µL of RNase-free water, 1µL of internal control (IC), and 2µL of enzymes for each reaction. An 8µl aliquot of RNA sample, 8µL of positive control (supplied in the kit), or 8µl of RNase-free water for negative control was added to the master mix, resulting in a final volume of 26 µL. Plates were then centrifuged at 2500 rpm for 5 seconds and analyzed on a CFX96 Touch Real-Time PCR from BioRad using the following conditions:

Reverse Transcription reaction one cycle: 50°C/20 minutes–95 °C/15 minutes.
PCR reaction 45cycles: 94 °C/15 seconds–58°C/30 seconds.
Gene amplifications were analyzed by FAM (E gene), HEX (IC), Cal Red 610 (RdRP), and Quasar 670 (N gene) fluorophores. Results were analyzed using a 2019-nCoV viewer from Seegene Inc. according to the manufacturer's instructions.

2.5. Quantification of cytokine expression by ELISA

Cytokine levels in a 100µL cell-free supernatant collected at different times post-infection were determined using Elabscience ELISA kits (Elabscience, Texas, USA). Briefly, a standard curve was generated for each cytokine using twofold serial dilutions of reference standards (supplied in each kit) tested in duplicate. The mean optical density (OD) value at 450nm for each dilution was used to generate a standard curve for each cytokine. To quantify each cytokine, 100µL of known standards (in duplicate) and samples (in duplicate) were incubated for 90 minutes at 37ºC. Post-incubation, the samples (and standards) were removed from each well, and plates were incubated with 100µL of biotinylated detection antibody. Post-incubation, plates were washed three times with 350µL of wash buffer and incubated with 100µL horseradish peroxidase (HRP) conjugated streptavidin solution, and the plates were incubated at 37ºC for 30 minutes. Post-incubation, the plate was washed five times with 350µL of wash buffer. A 90µL aliquot of substrate reagent was added to each well and incubated for 15 minutes at 37ºC, and the reaction stopped with a 50µL aliquot of stop solution. The OD values at 450nm were determined after 15 minutes using a SpectraMax Plus384 reader.

2.6. Statistical analysis

Statistical analysis and graphical presentation of results were performed using GraphPad Prism version 9.2.0. Differences in viral RNA and cytokine levels were determined using the analysis of variance (ANOVA) test, and a p-value of less than 0.05 was considered statistically significant. The viral load was determined at each time point from one sample while each cytokine level was tested at each time point using two biological replicates.

3. Results

3.1. Cytotoxicity assay

The study aimed to determine whether plant extracts have the potential to considerably reduce viral RNA load and modulate cytokine levels associated with the cytokine storm that occurs in some COVID-19 patients. H1299-hACE2-E3 cells were chosen as they are permissive to SARS-CoV-2 due to the expression of ACE-2 receptors. The cells were incubated with dilutions of Phela and the four individual components of Phela, including *Gladiolus dalenii*, *Senna occidentalis, Rotheca myriciodes,* and *Clerodendrum glabrum*, to determine suitable concentrations that could be used to treat the cells without causing cytotoxic effects. The cells were incubated with plant extracts prepared from stock solutions of 2mg/mL and diluted twofold from 1600µg/mL to 100µg/mL, then 80µg/mL to 10µg/mL in cell culture media. Dilutions between 400µg/mL and 1600µg/mL of plant extracts were found to be toxic to the cells, resulting in cell death. MTS assays showed that Phela exhibited dose-dependent cytotoxicity on h1299-hACE2-E3 cells with CC_{50} values of 400µg/mL ± 200µg/mL, while three of the components of Phela, including *Clerodendrum glabrum*, *Rotheca myriciodes*, and *Senna occidentalis*, exhibited a CC_{50} of 200 ± 100µg/mL (Table 3.1) (Figure 3.1) respectively. The exception was *Gladiolus dalenii,* whose CC_{50} was undetermined. Treatment of cells with *Gladiolus dalenii* resulted in more than 50% cell death, even at the lowest concentration, and thus was excluded from the infection studies. A final concentration of 100µg/mL was selected as a suitable non-cytotoxic concentration to assess the ability of plant extracts.

Table 3.3: Cytotoxicity of plant extracts against h1299-hACE2-E3 cells

Plant extract	**CC_{50} (µg/mL)**
Clerodendrum glabrum	200 ± 100
Rotheca myriciodes	200 ± 100
Gladiolus dalenii	na
Senna occidentalis	200 ±100
Phela	400 ± 200

CC_{50}: Concentration inhibiting cell viability by 50%

na: concentration not available

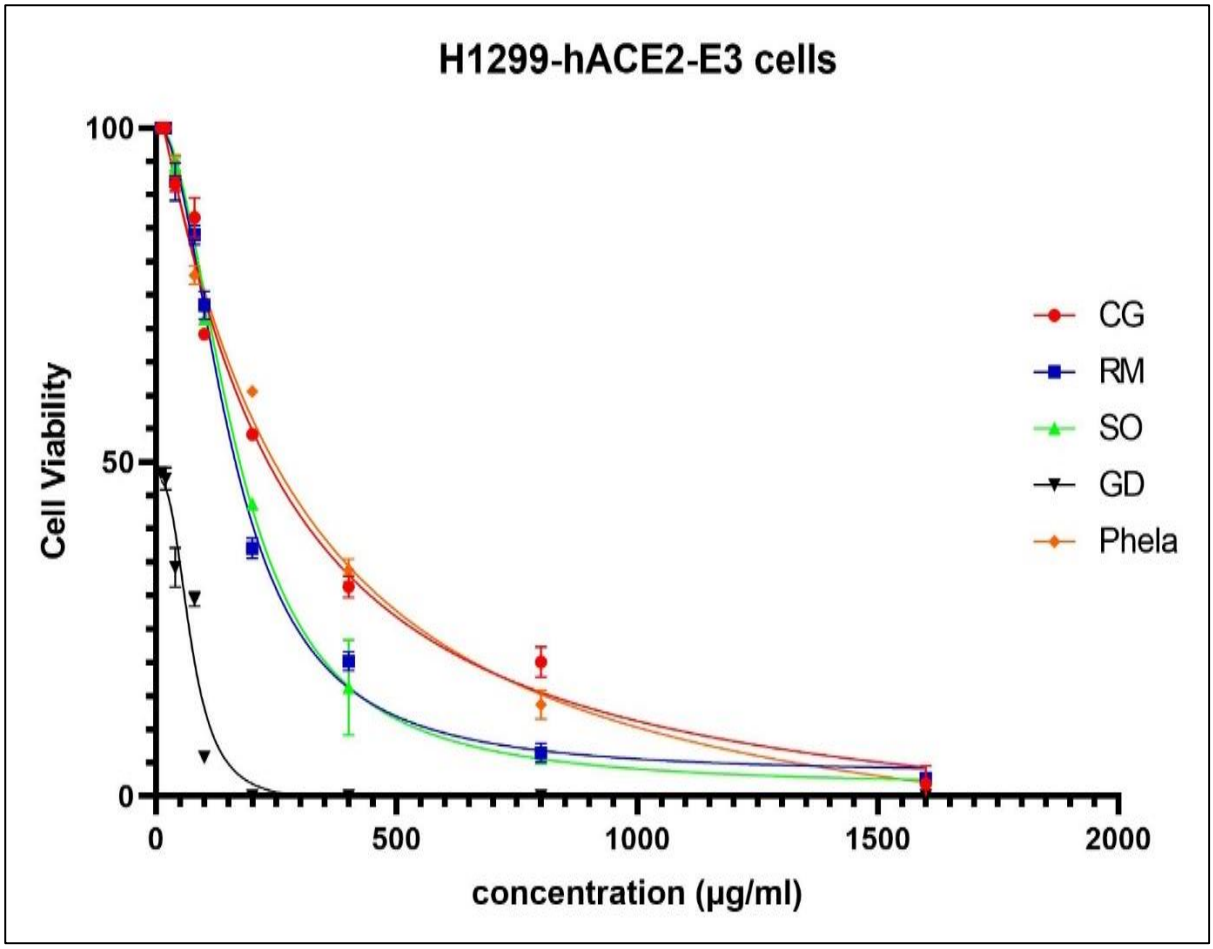


Figure 3.2: Cell viability dose-response curve. H1299-hACE2-E3 cells were treated with plant extracts from 1600, 800, 400, 200, 100, 80, 40, 20, to 10µg/mL for 72 hours, and cell viability was evaluated using the MTS assay. Results were represented as mean ± SD of two independent experiments and are expressed as relative values compared to the untreated cells.

3.2. Infection of h1299-hACE2-E3 cells with SARS-CoV-2 Omicron variant

Growth curves of cells infected with SARS-CoV-2 and treated with plant extracts were determined by first treating cells with plant extracts and incubation for 30 minutes before infecting with 100 $TCID_{50}$ of SARS-CoV-2 and by infecting cells with 100 $TCID_{50}$ before treatment with plant extracts. Virus replication was quantified by RT-qPCR from RNA extracted from cell supernatant at 12, 24, 48, and 72 hours post-infection. Replication curves from treated cells were compared to those obtained from infected, untreated cells. Results were represented as cycle threshold (Ct) values, where a low Ct value indicates a high viral load and a high Ct value indicates a low viral load. The assays target three genes, including envelope (E), RdRP, and the N gene. All samples were positive for at least two of three viral targets (Appendix A). Figure 3.2 A-C

represent the viral loads at different time points for the E, RdRP, and N gene. Appendix A shows the Ct values obtained at different time points after infection and treatment for each gene. Results show no statistical significance in viral load between cells treated with plant extracts compared to the virus-infected control. Post-treatment of cells with Phela, although not statistically significant, resulted in a higher Ct value than the rest of the treated samples and the virus-infected control, suggesting a possible delay in virus replication. Cells post-treated with Phela had a higher Ct value, 31.49, 34.22, and 32.42 for E, RdRP, and N gene, respectively, at 12h post-infection compared to others which had Ct values in the range of 12 to 19. Additionally, pretreatment of cells with Phela shows a viral load reduction at 72h post-infection for the E and N genes, which was not seen in other samples. The results show that although not significant, the Phela may potentially interfere with viral load.

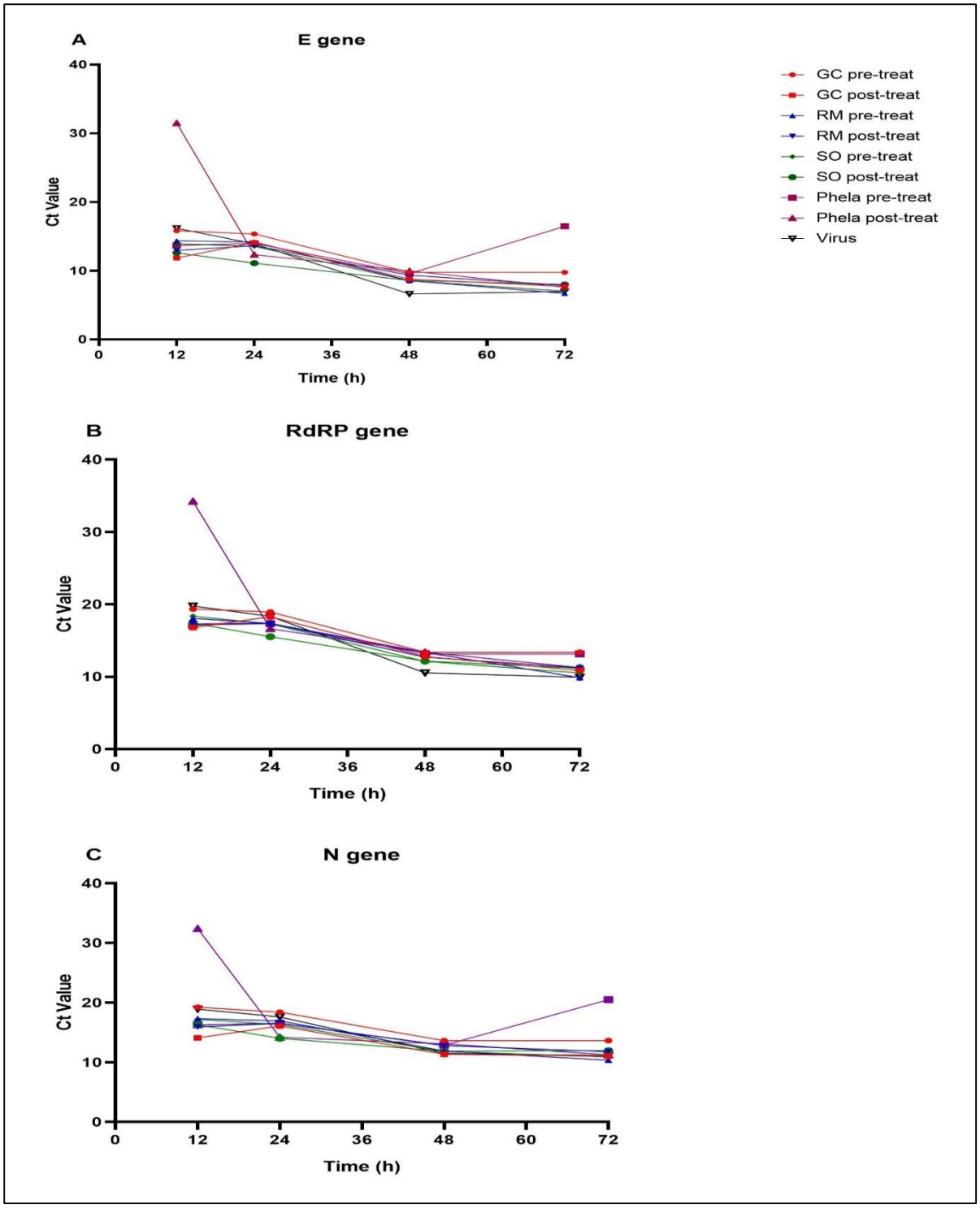


Figure 3.2: SARS-CoV-2 growth curves pretreatment and post-treatment with plant extracts. H1299-hACE2-E3 cells were incubated with 80µg/mL of *Clerodendrum glabrum*, *Rotheca myriciodes*, *Senna occidentalis*, and Phela for 30 minutes before infection or h1299- hACE2-E3 cells were infected with SARS-CoV-2 Omicron variant before treatment with 80µg/mL of *Clerodendrum glabrum*, *Rotheca myriciodes*, *Senna occidentalis*, and Phela. RNA was extracted at 12, 24, 48, and 72 hours, and viral loads were evaluated by qRT-PCR.

3.3. Cytokine release post-infection with SARS-CoV-2 infection

The study was designed to assess the potential of Phela and its components to modulate immune responses by analyzing cytokine levels associated with cytokine storms. Cytokine storms, which involve the overproduction of certain cytokines in response to viral infections, have been associated with the severe symptoms observed in COVID-19 patients (Lavillegrand et al., 2021). The levels of IL-1β, IL-2Rα, IL-6, IL-10, TNF-α, and IFN-γ were assessed in cells treated with plant extracts pre- and post-infection and compared to untreated cells and uninfected cells at 12, 24, 48, and 72 hours post-infection.

Severe SARS-CoV-2 infection is strongly associated with the release of TNF-α in infected patients. An increase in extracellular TNF-α, IL-1β, and IL-2Rα was detected in SARS-CoV-2 Omicron-infected cells (Figures 3.3-3.5). At 48 and 72 hours post-infection, levels of TNF-α were significantly higher in virus-infected untreated cells compared with infected cells treated with plant extracts, both pretreatment and post-treatment (Figure 3.3). Results also show that the levels of TNF-α were significantly lower in post-treated cells compared to pretreated cells at 72 hours post-infection, except for cells treated with *Clerodendrum glabrum*. Results show a delay in TNF-α release in cells pretreated with *Clerodendrum glabrum* and *Rotheca myriciodes*. Detection of TNF-α was only evident 24 and 48 hours post-infection for RM and *Clerodendrum glabrum*, respectively (Figure 3.6A). In contrast, post-treatment delayed TNF-α production in all treated cells, with significantly higher levels detected at 48 ($p=0.0001$) and 72 ($p=0.0001$) hours post-infection (Figure 3.6B). A comparison of TNF-α levels in the supernatant of cells treated with different plant extracts showed that *Clerodendrum glabrum* and *Rotheca myriciodes* had the lowest levels detected compared to *Senna occidentalis* and Phela at 48 and 72 hours post-infection in the pretreatment group (Figure 3.6A). In contrast, in the post-treatment group, TNF-α levels were similar at 48 hours post-infection in all groups (Figure 3.6B), whereas TNF-α levels in *Clerodendrum glabrum*-treated cells were significantly higher than the other plant extracts at 72 hours post-infection.

Similarly, IL-1β levels were significantly higher 48 and 72 hours post-infection in virus-infected control cells compared to cells treated with plant extracts, except for Phela at 72 hours post-infection, where the pretreatment group was higher compared to the control (Figure 3.4). Similar trends were noted between pretreatment and post-

treatment, except for when treated with Phela, which had significantly higher levels at 24 (p=0.0001) and 72 (p=0.0001) hours post-infection compared with virus-infected control cells (Figure 3.7A). In the post-treatment group, levels of IL-1β were comparable between cells treated with the different plant extracts and significantly lower compared to the virus-infected untreated cells at 48 (p=0.0001) and 72 (p=0.0001) hours post-treatment.

In contrast, higher levels of IL-2Rα were seen compared to TNF-β and IL-1β. However, virus-infected control cells still had significantly higher cytokine levels than those treated with plant extracts (Figure 3.5). Cytokine levels in cells pretreated with plant extracts compared to virus-infected control differed 48 hours post-infection (range of p=0.01 and 0.0001) (Figure 3.8A). At 72 hours post-infection, all pretreated cells had significantly lower levels of IL-2Rα compared to the virus-infected control cells (p=0.0001) (Figure 3.8A). Significantly higher levels of IL-2Rα were evident in virus-infected control cells compared to cells post-treated with plant extracts at 48 (p=0.0001) and 72 (p=0001) hours post-infection (Figure 3.8B). Similarly to TNF-α and IL-1β, there were differences in levels of IL-2Rα in pretreated cells, whereas levels were comparable in post-treated cells. IFN-γ was not detected from cells infected with SARS-CoV-2 Omicron virus and treated with plant extracts or from uninfected control cells. However, increased levels of IFN-γ were detected at 48 and 72 hours post-infection in virus-infected, untreated cells (Figure 3.9). IL-6 and IL-10 were not detected during infection with SARS-CoV-2.

The results show that the timing of treatment (pre- or post-treatment) appears to influence the level of cytokine release, with post-treatment showing better promise, except in the case of *Clerodendrum glabrum*, where pretreatment had more influence. The results suggest that Phela and its components might have a potential role in modulating the immune response during SARS-CoV-2 infection. Specifically, the plant extracts seem to reduce the release of proinflammatory cytokines like TNF-α, IL-1β, and IL-2Rα, which are implicated in the cytokine storms observed in severe COVID-19 cases. These results show that the plant extracts may notably reduce the three key cytokines, TNF-α, IL-1β, and IL-2Rα, associated with cytokine storms triggered by SARS-CoV-2 infection. However, further research is needed to understand the mechanisms involved in immune-modulating.

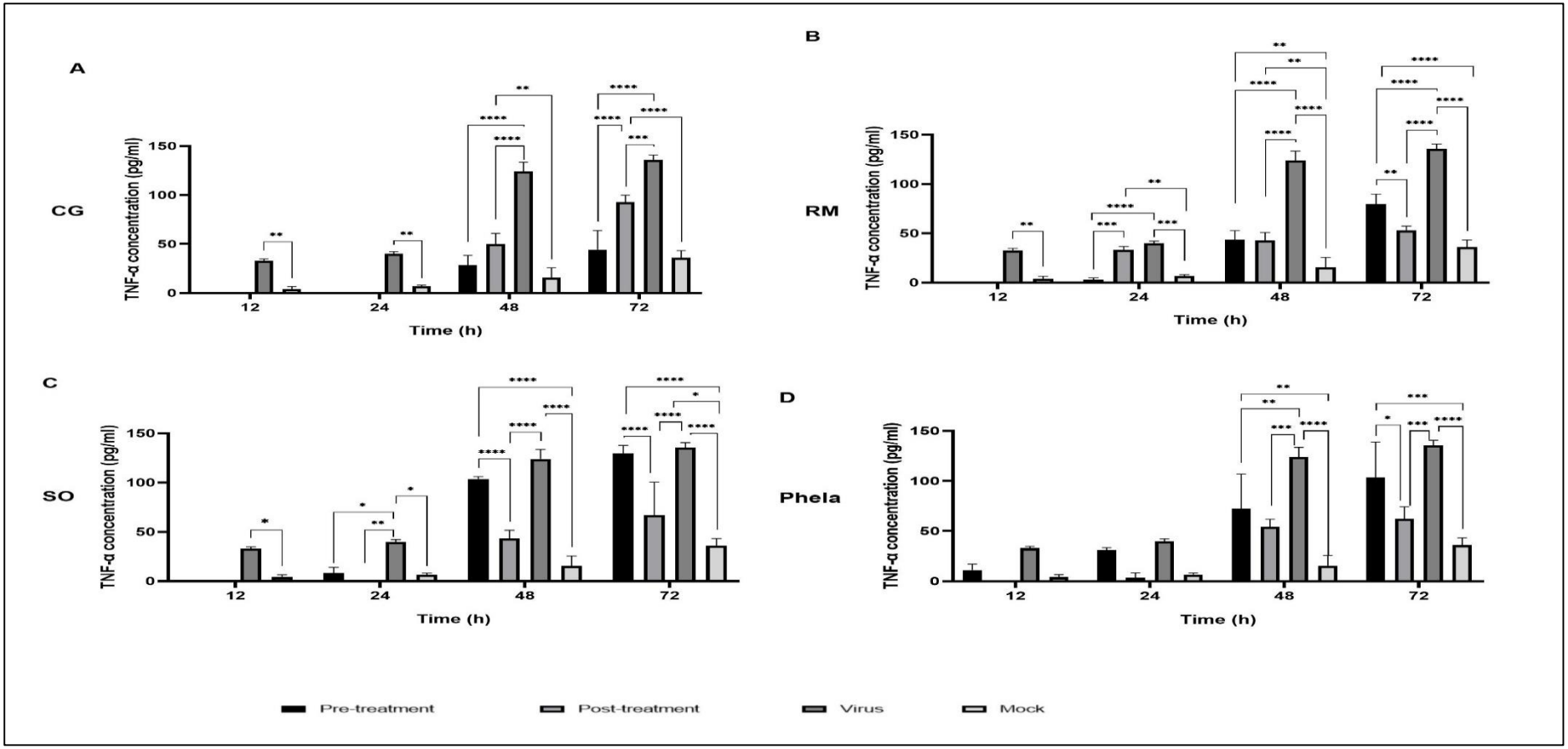


Figure 3.3: Cytokine levels in SARS-CoV-2 Omicron infected h1299-hACE-2-E3 cells. Cells were infected with SARS-CoV-2 pre- and post-treatment with plant extracts. Cell supernatant was collected at 12, 24, 48, and 72 hours post-infection, and extracellular release of TNF-α quantified by ELISA in cells treated with. A. *Clerodendrum glabrum*. B. *Rotheca myriciodes*. C. *Senna occidentalis*. D. Phela. ****p<0.0001, *** p<0.001, **p< 0.01, *p<0.05

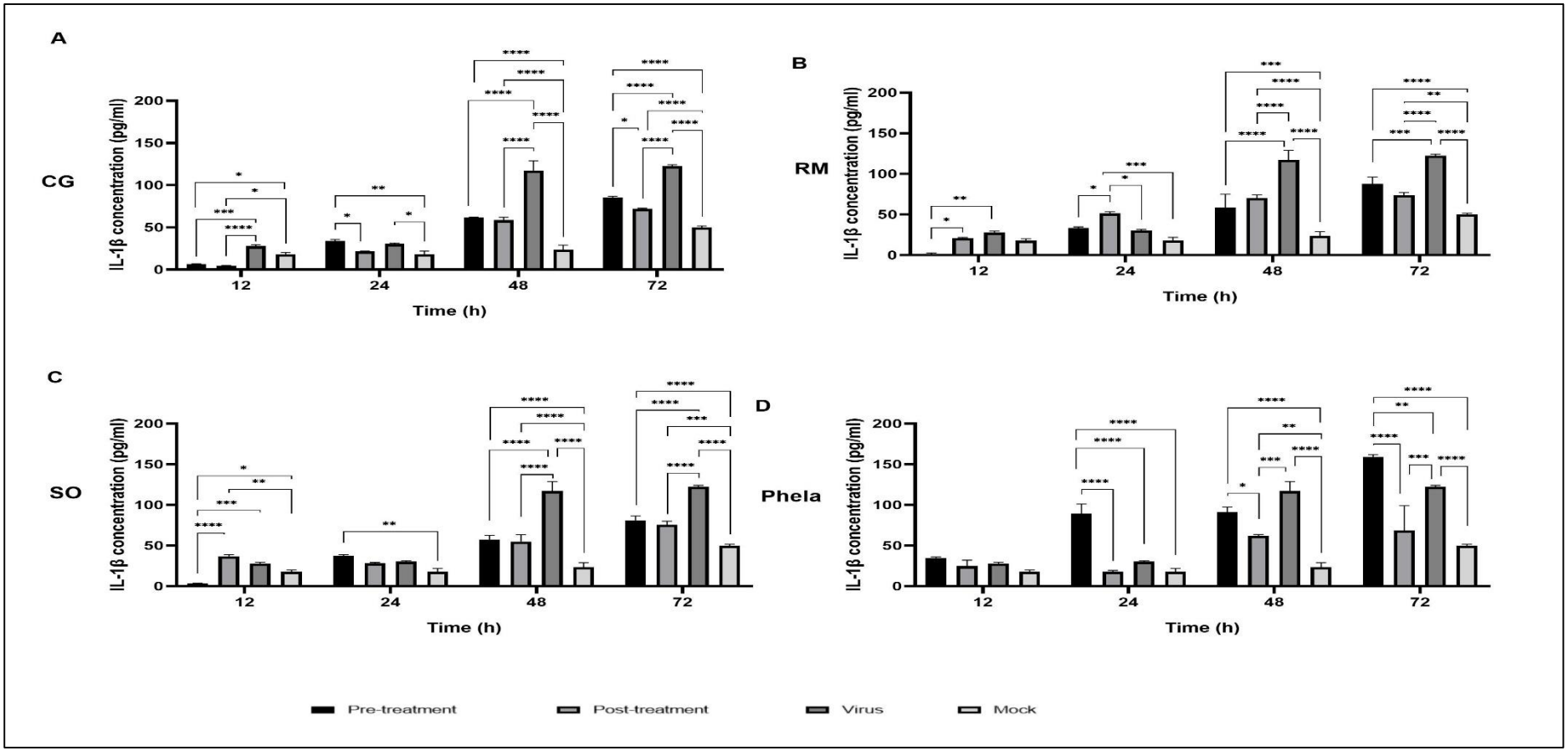


Figure 3.4: Cytokine levels in SARS-CoV-2 Omicron infected h1299-hACE-2-E3 cells. Cells were infected with SARS-CoV-2 pre- and post-treatment with plant extracts. Cell supernatant was collected at 12, 24, 48, and 72 hours post-infection, and extracellular cytokine release of IL-1β quantified by ELISA in cells treated with. A. *Clerodendrum glabrum*. B. *Rotheca myriciodes*. C. *Senna occidentalis*. D. Phela. ****p<0.0001, *** p<0.001, **p< 0.01, *p<0.05

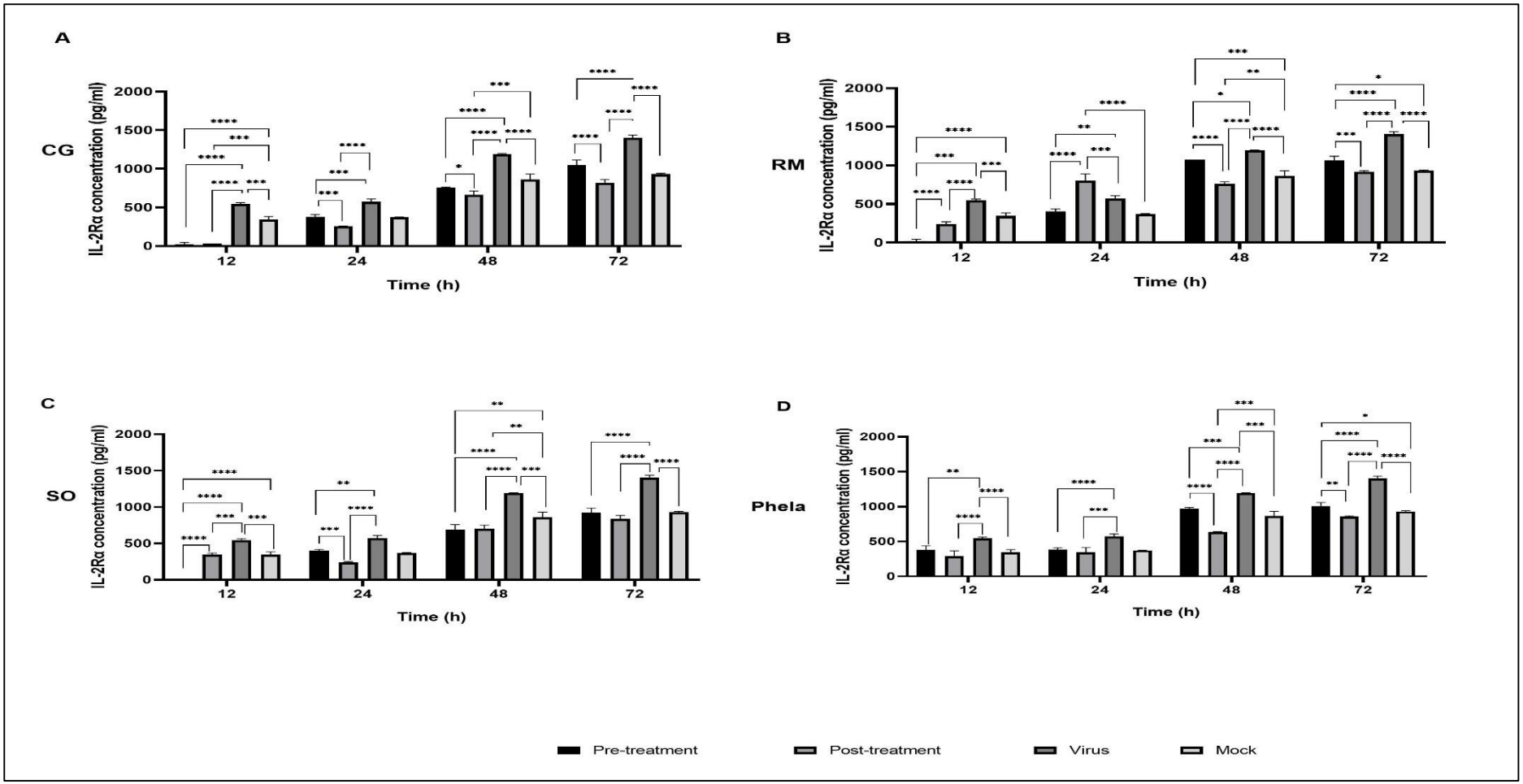


Figure 3.5: Cytokine levels in SARS-CoV-2 Omicron infected h1299-hACE-2-E3 cells. Cells were infected with h1299 pre-and post-treatment with plant extracts. Cell supernatant was collected at 12, 24, 48, and 72 hours post-infection, and extracellular cytokine release of IL-2Rα were quantified by ELISA in cells treated with. A. *Clerodendrum glabrum*. B. *Rotheca myriciodes*. C. *Senna occidentalis*. D. Phela. ****p<0.0001, *** p<0.001, **p< 0.01, *p<0.05

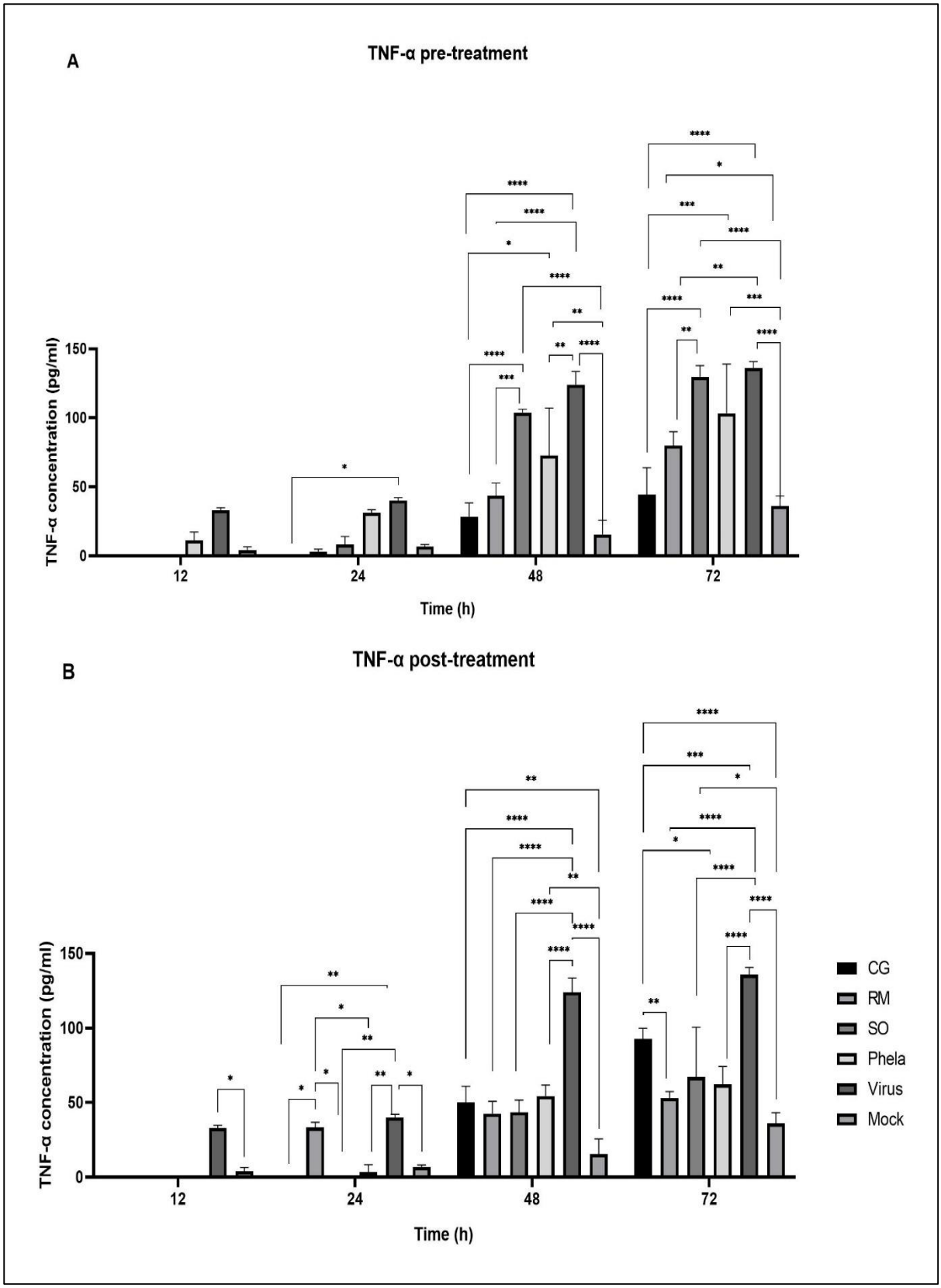


Figure 3.6: TNF-α levels in cells pretreated and post-treated with plant extracts and infected with SARS-CoV-2 Omicron. A: Cells pretreated with plant extracts before infection. B: Cells treated post-infection. Cell supernatant was collected 12, 24, 48, and 72 hours post-infection, and TNF-α levels quantified by ELISA. ****p<0.0001, *** p<0.001, **p< 0.01, *p<0.05

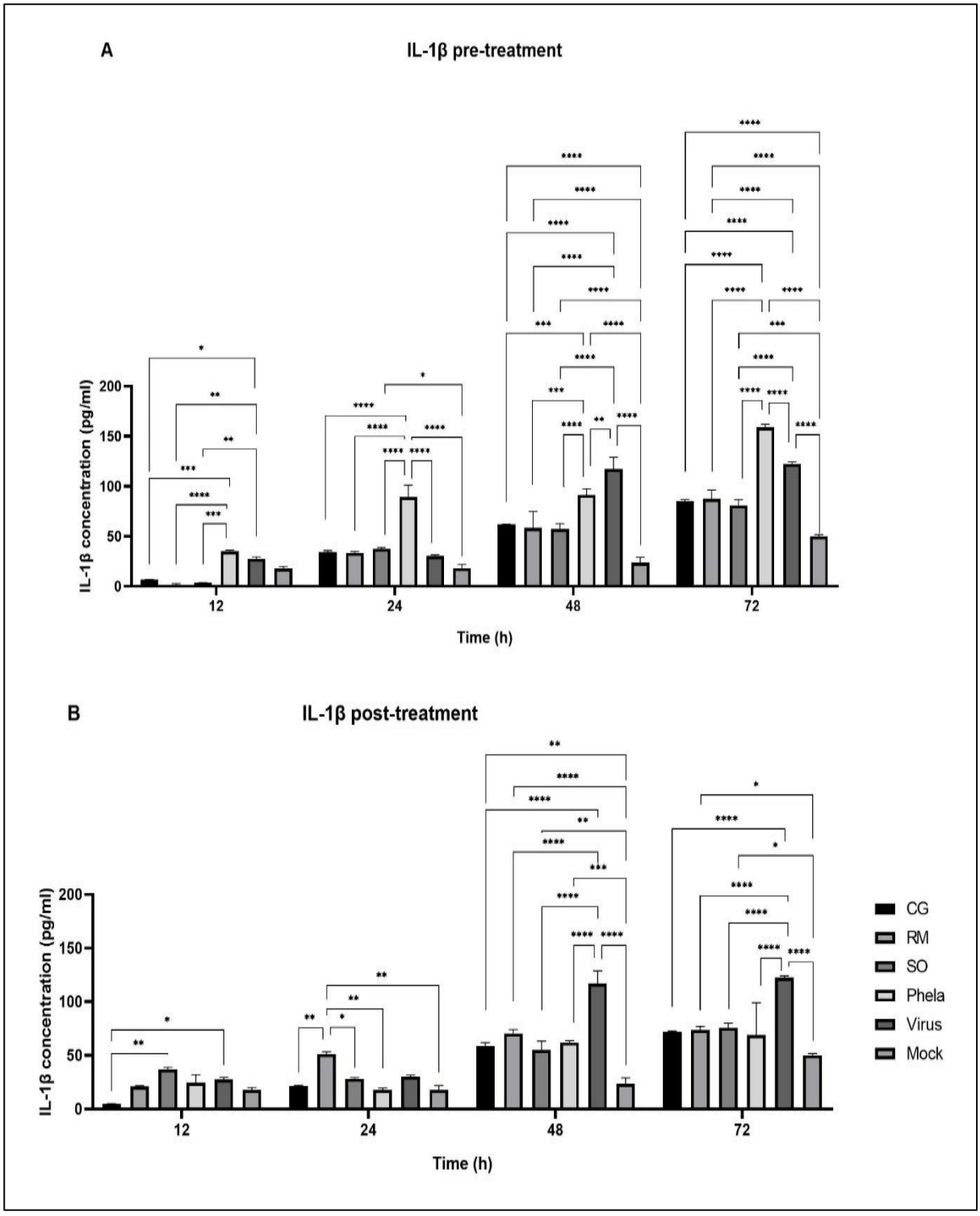


Figure 3.7: IL-1β levels in cells pretreated and post-treated with plant extracts and infected with SARS-CoV-2 Omicron. Cell supernatant was collected 12, 24, 48, and 72 hours post-infection, and IL-1β levels quantified by ELISA. A: Cells pretreated with plant extracts before infection. B: Cells treated post-infection. ****p<0.0001, *** p<0.001, **p< 0.01, *p<0.05

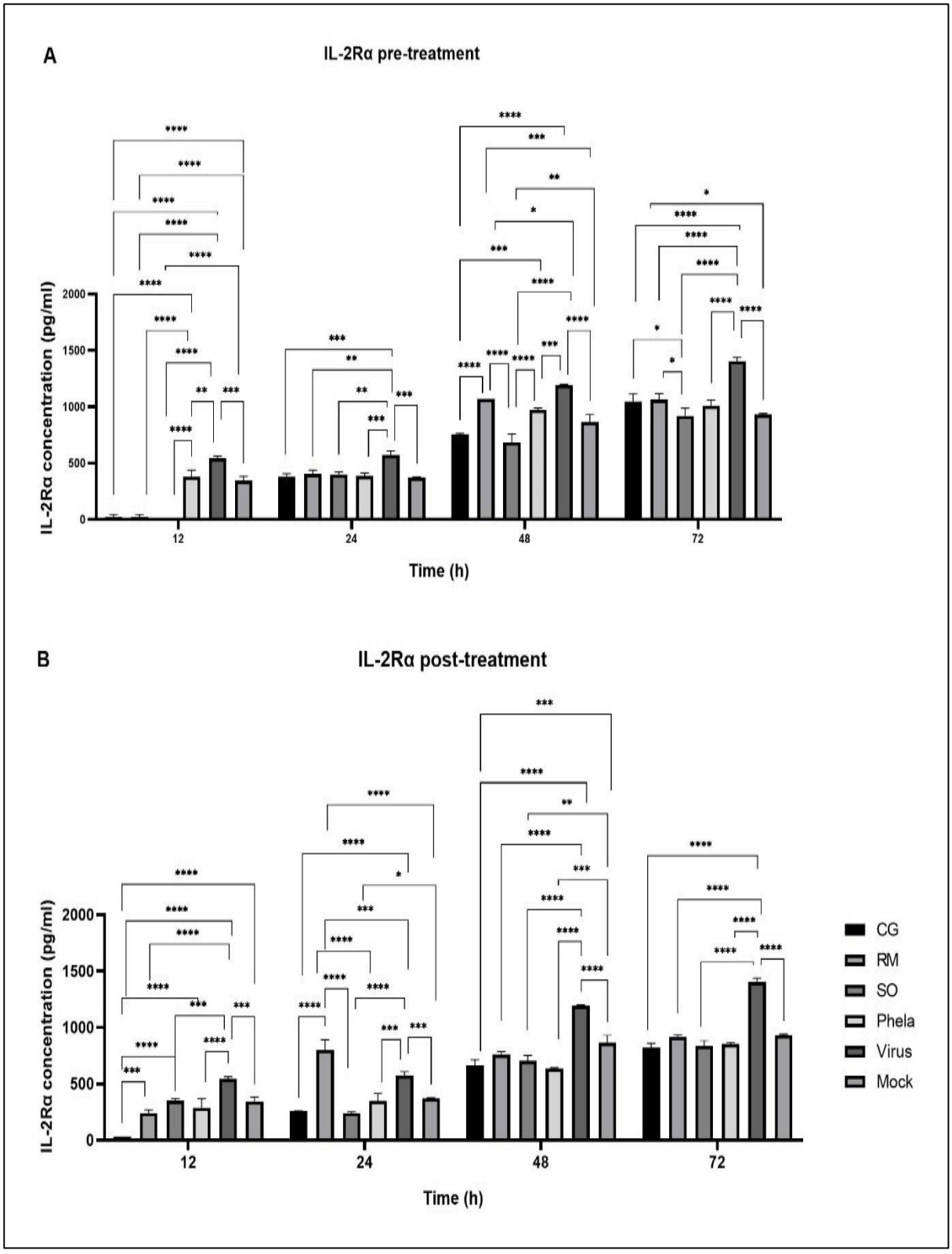


Figure 3.8: IL-2Rα levels in cells pretreated and post-treated with plant extracts and infected with SARS-CoV-2 Omicron. Cell supernatant was collected 12, 24, 48, and 72 hours post-infection, and IL-2Rα levels quantified by ELISA. A: Cells pretreated with plant extracts before infection. B: Cells treated post-infection. ****p<0.0001, *** p<0.001, **p< 0.01, *p<0.05

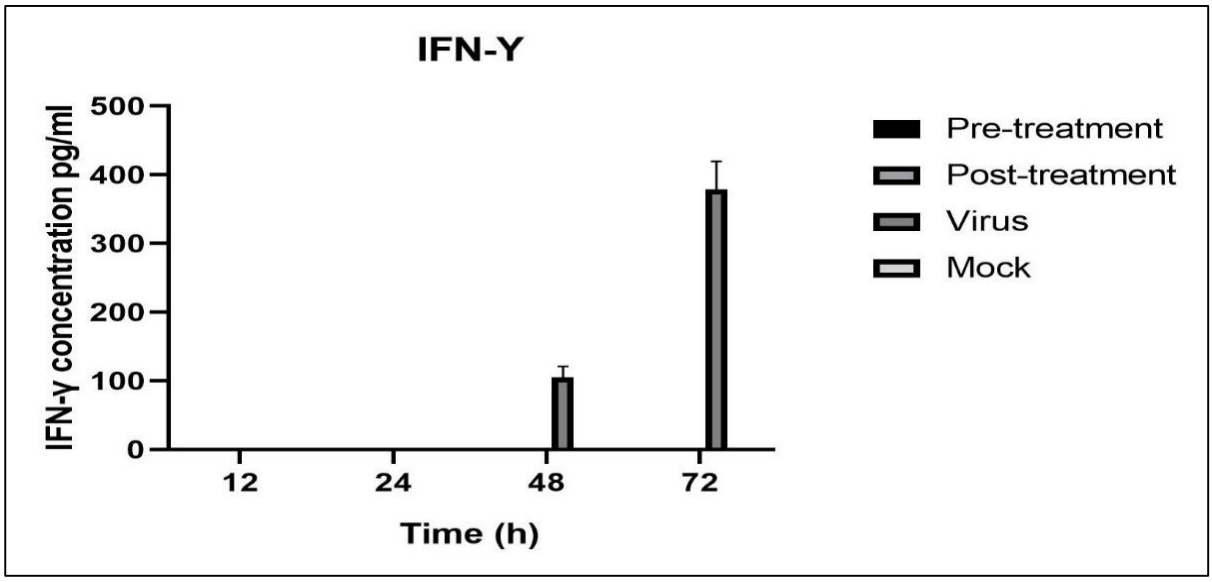


Figure 3.9: IFN-γ levels in cells pretreated and post-treated with plant extracts and infected with SARS-CoV-2 Omicron. Cell supernatant was collected 12, 24, 48, and 72 hours post-infection, and extracellular release of IFN-γ quantified by ELISA.

4. Discussion

The current vaccines approved for use have been shown to reduce disease severity and lower fatality rates. However, novel therapeutics are still required for treatment and management of disease. This is important as possible breakthrough infections can occur in previously infected and vaccinated individuals. In addition, there is a possibility that new variants may emerge or existing variants may evolve to evade the immune response. Traditional medicinal plants have been used previously to treat various respiratory infections, as they are believed to have promising antiviral capacities (Abd-Alla et al., 2019; Mehrbod et al., 2019; Thabti et al., 2020). Different countries have tested potential indigenous plants for immune-modulating capabilities against SARS-CoV-2 (Nie et al., 2021; Tang et al., 2021; Flórez-álvarez et al., 2022) and compounds in plant extracts with the potential to have antiviral activity against SARS-CoV-2 have been identified using in silico molecular docking (Maurya et al., 2022). In silico molecular docking is a computational method used in drug discovery and research to predict binding interactions between small molecules and target proteins in order to understand their therapeutic effects. This can help in rapid identification of promising drug candidates against COVID-19. Traditional plants that have previously been shown to be efficient against viral infection are one approach for

identifying cost-effective treatments during outbreaks/pandemics when no effective treatments are available.

This study evaluated the activity of Phela and its components against SARS-CoV-2 using mammalian cell lines overexpressing the ACE-2 receptor. Phela is under development as an immune booster in South Africa, with recent studies showing its effectiveness in the treatment of gram-positive *Staphylococcus aureus* (Das et al., 2022) and possible use for the treatment of Alzheimer's disease (Das et al., 2020). This study aimed to assess the effect on viral load pre-and post-treatment with plant extracts and cytokine release associated with cytokine storms after treatment with the plant extracts. Based on Ct values for viral loads, treatment of cells with plant extracts did not appear to inhibit viral replication. There was no statistical significance in Ct values between cells treated with plant extracts and the virus-infected control cells, suggesting that the plant extracts may not have the ability to inhibit SARS-CoV-2 replication. However, the concentration of the plant extracts used may have also been too low to impact virus replication. Although not significant, post-treatment with Phela resulted in higher Ct values compared to virus-infected control cells and cells treated with other plant extracts. Post-treatment of cells with Phela resulted in Ct values of 31.49, 34.22, and 32.42 for the E, RdRp, and N genes, respectively (Appendix A). Ct values of cells treated with the components of Phela pre- and post-treatment resulted in Ct values between 12 and 19, and virus-infected control cells had Ct values between 16 and 19. These results may suggest that post-treatment with Phela caused a delay in viral RNA replication, suggesting that post-treatment of cells with Phela may have potentially interfered with viral replication.

There were clear differences in levels of cytokines due to treatment with plant extracts. Multiple studies have reported on the dysregulated production of inflammatory cytokines in patients with severe COVID-19 disease (Lucas et al., 2020; Vanderbeke et al., 2021; Sun et al., 2022). Release of these cytokines has been shown to be an essential indicator of the severity of disease for SARS-CoV-2, associated mainly with organ failure rather than viral load. Previous studies have also associated the release of IL-6, IL-1β, and TNF-α with the severity of the disease (Ashrafzadeh-Kian et al., 2022; Jing et al., 2022). In this study, key inflammatory cytokines, including IL-1β, IL-2Rα, IL-6, IL-10, TNF-α, and IFN-γ, that have been associated with cytokine storm were included in our analysis. Some cytokines, including TNF-α, IL-1β, and IL-2Rα,

showed significantly higher release in virus-infected cells than those treated with plant extracts. The pretreatment of cells with plant extracts significantly reduced TNF-α at 48 hours post-infection in cells pretreated with *Clerodendrum glabrum*, *Rotheca myriciodes*, and Phela, but not *Senna occidentalis* before infection. Moreover, at 72 hours post-infection, *Clerodendrum glabrum* and *Rotheca myriciodes* demonstrated a significant reduction in TNF-α release, while *Senna occidentalis* and Phela did not show the same effect. Pretreatment significantly reduced IL-1β in cells treated with *Clerodendrum glabrum*, *Rotheca myriciodes*, *Senna occidentalis*, and Phela at 48 hours post-infection. However, a significant reduction was seen only with *Clerodendrum glabrum*, *Rotheca myriciodes*, and *Senna occidentalis* at 72 hours post-infection, with Phela being significantly higher than the control of virus-only infected cells. Post-treatment of cells with immune modulators resulted in a significant reduction of IL-1β, IL-2Rα, and TNF-α at 48 and 72 hours post-infection of Phela and all its components, suggesting a potential role of these plant extracts in modulating the immune response by reducing TNF-α release during SARS-CoV-2 infection. IFN-γ was not detected in cells treated with Phela, all its components, and the uninfected cells. In contrast, high levels of IFN-γ were detected in h1299-hACE2-E3 virus-infected untreated cells at 48 and 72 hours post-infection. Results also show a better response post-treatment than pretreatment, suggesting a role in managing cytokine storms associated with severe symptoms of COVID-19 disease. The results show the complexity of innate immune response to viral infections and the potential of Phela and components of Phela in modulating innate immune responses. Understanding the underlying mechanisms through which these compounds interact with the immune system could provide valuable insights into possible treatments for SARS-CoV-2 and related viruses to manage the dysregulated immune responses in severe cases. Previous studies have associated IL-6 with severity of disease (Lavillegrand et al., 2021). However, in this study, IL-6 and IL-10 were not detectable in cells treated with plant extracts or the controls. This could possibly be due to the ELISA methods not being sensitive enough to detect low levels of these cytokines. Another reason for the IL-6 being undetectable could be the early sampling post-infection, the timing of sample collection in relation to the progression of the infection may influence the detectability of the proinflammatory cytokine. Majority of individuals in which high levels of IL-6 is detected are usually in the severe stage of the disease, with disease

severity usually occurring five days post-infection. In our case, sampling was only performed for up to 72 hours in infected cells.

In summary, Phela and three out of its four components demonstrated a potential to reduce the upregulation of proinflammatory cytokines associated with severe SARS-CoV-2 disease. However, treating cells with plant extracts did not result in reduced viral loads post-infection with SARS-CoV-2 Omicron variant, suggesting that they may have a limited role in reducing viral load. Whether the observed effects could be relevant for use in humans needs to be further investigated. These results warrant further studies on the effect of traditional medicinal plants on modulating cytokine release during viral infections. The limitation of the study is that the small design and sample number warrant repeating the experiments with more samples and additional parameters and using gene expression studies or microarrays to further characterize the effect on the release of cytokines. Additionally, the results obtained in *in vitro* studies are not readily transferrable to a clinical setting to test in humans. However, they do provide the potential these extracts may have against SARS-CoV-2. Cytokine storms are not unique to SARS-CoV-2 and can occur in response to various viral infections and other inflammatory conditions. If Phela or its components prove effective in modulating cytokine responses, they could have broader applications in treating diseases characterized by excessive immune activation. These findings provide valuable insights into understanding the immunological aspects of viruses and pave the way for further exploration of plant-based therapies for managing COVID-19 and related inflammatory responses.

Authors Contribution

Litabe M: Investigation, methodology, data curation, formal analysis, writing- original draft, reviewing and editing

Matsabisa M: Plant extract acquisition, writing- review, and editing

Burt FJ: Methodology, supervision, writing– review and editing, and funding acquisition

Conflict of Interest:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 7
Conclusion and Future Perspectives

Several lethal CoVs have emerged in the past two decades, including SARS-CoV, MERS-CoV, and the recent SARS-CoV-2. CoVs are a perfect example of how globalization and travel accessibility worldwide can lead to the rapid spread of infectious diseases. Evolutionary analysis provides evidence that these viruses likely originated from bats and passed to humans through an intermediate host. SARS-CoV emerged in Guangdong, China, in mid-November 2003 (Fouchier et al., 2003). By late February 2003, cases were reported in Hong Kong, and the virus began to spread to other countries, including Canada, Singapore, and Vietnam (CDC, 2003) and subsequently spread to 29 countries, resulting in over 8000 cases and a case fatality of 9%. MERS-CoV emerged in 2012 in Saudi Arabia (Osterhaus et al., 2012). The virus was thought to be concentrated in Saudi Arabia, but later cases were reported in other countries, such as South Korea and the United Arab Emirates (Rabeeah et al., 2013; Cowling et al., 2015). Since December 2019, SARS-CoV-2 has resulted in widespread devastation globally, and although WHO declared that the Public Health Emergency for International Concern for COVID-19 was over on 5 May 2023, COVID-19 remains a significant threat and continues to cause disease.

SARS-CoV-2 infection and vaccination result in both humoral and cellular immunity, which play an essential role in protection and clearance of the virus (Almendro-Vázquez et al., 2021; Rose et al., 2022). Neutralizing antibodies and T-cell responses are believed to be a correlate of protection against SARS-CoV-2 (Zost et al., 2020; Primorac et al., 2022). This study aimed to investigate SARS-CoV-2 antibody kinetics and determine the duration of immunity in previously COVID-19-infected patients. It is essential to investigate antibodies post-infection and vaccination as they help determine the proportion of the population that has developed immunity against SARS-CoV-2 while monitoring the progress towards achieving herd immunity. Although the exact correlates of protection have not been fully defined, and the role of antibodies in immunity is not clear, antibody studies provide a useful biomarker of infection and/or vaccination. Many serological tests for detecting IgA, IgM, and IgG antibodies were developed, which differ in sensitivity and specificity (Beavis et al., 2020; Jääskeläinen et al., 2020; Hardy et al., 2021). However, access to these tests

at the beginning of the pandemic was challenging and expensive, making it important to develop in-house serological assays. This offers an added advantage, as in-house assays are cheaper. Additionally, it reduces dependency on external suppliers, as the heightened need for serological assays during a pandemic can make it difficult to access the kits.

Therefore, two in-house serological assays, ELISA and IFA, were developed and validated to detect anti-S IgG antibodies in a South African population (Matefo et al., 2022). Validating assays in populations where they will be used is essential, as the specificity and sensitivity may vary. The S protein is a primary target for neutralizing antibodies and the key protein used in vaccine developments (Yang and Du, 2021), making it a target protein for serological assays. Eighty-nine serum samples were collected from COVID-19 PCR-confirmed patients and used to validate the assay. A 100 prepandemic samples were used as a negative panel to determine a suitable cutoff for differentiating positive from negative samples. The assays showed high specificity, 96% and 100% for ELISA and IFA, respectively. The assays were also compared to commercially available SAPRHA approved assay, where the in-house ELISA showed a higher positive predictive value (PPV) of 95.8%, while the Roche assay was 89.6%, and the commercial lateral flow was 93.9%. The two in-house assays also detected IgG antibodies in 62 samples collected from waves in which new variants were circulating, showing that despite mutations of the SARS-CoV-2 S protein, the assay was still sensitive to detect IgG antibodies in circulating variants likely due to targeting of multiple epitopes within the S protein. The evaluation confirmed the application of these assays for detection of IgG antibodies and surveillance within the South African population.

Subsequent to evaluation, the in-house assays were used to test for IgG antibodies and determine the duration and kinetics of antibodies in 100 individuals over two years, between March 2021 and March 2023. A total of 244 samples were collected and grouped based on the days post-symptom onset that the sample was collected to determine the duration of anti-S IgG antibodies in recovered individuals. Results showed that IgG antibodies were significantly higher directly after infection, between 1-180 days post-onset, and gradually declined significantly, although still detectable at low levels, indicating waning of antibodies with time. Three sequential samples were collected from 62 individuals to determine the longevity and trajectory of IgG

antibodies over time. Overall, 90.3% (56/62) of the individuals still had detectable antibodies two years post-symptom onset, with vaccinated individuals having significantly higher IgG antibodies. The results suggest that there is longevity of antibodies, which may have been boosted by reinfection, possibly asymptomatic, and/or vaccination. The study also shows that the dynamics of anti-S IgG antibodies vary among individuals; most individuals display declining (61.3%) antibody titers with time, and only a smaller proportion displayed an increase in antibody detected (8.1%) or remained unchanged (6.5%) or fluctuated (20.9%) over time. One study showed that the stability of antibodies may be due to a higher baseline of antibodies developed at the beginning of infection, and individuals with a more severe disease develop a higher titer of antibodies that remains stable for longer (Haveri et al., 2021). The severity of disease was not available for this study. The duration of detectable antibody indicates the inappropriateness of serology for diagnosis. However, it is a useful tool for surveillance.

The long-term application of serological assays will depend on continued reactivity against mutating viruses and detection of responses against emerging variants. Like most RNA viruses, SARS-CoV-2 has mutated and changed over time, with the mutations influencing characteristics, including transmissibility, disease severity, and vaccine efficacy. Thus, variant classification is essential for alerting countries regarding new emerging variants that could impact epidemiological situations, diagnostic assays, including molecular assays, and the efficacy of vaccines. The detection of IgG antibodies is a useful biomarker. However, neutralizing antibody responses are likely more important for protection against the virus. Hence, the importance of monitoring neutralizing antibody efficacy against emerging variants, which could indicate potential for vaccine breakthrough and/or reinfection. There are different categories in which the emerging variants are classified by the WHO, including variants under monitoring, variants of interest, or variants of concern. In our study, the Ancestral strain and two variants of concern, Delta and Omicron variants, were isolated from residual diagnostic qRT-PCR COVID-19 positive samples and used for microneutralization assays to determine the duration and endpoint titer of the 100 individuals recruited for the study. The aim was to determine the duration and cross-neutralization ability of neutralizing antibodies against the Ancestral strain, Delta, and Omicron in samples collected between March 2021 and March 2023. Neutralizing

antibodies have been identified as a key correlate of protection against SARS-CoV-2 (Sui et al., 2021; Lingas et al., 2023). Although it is unclear what level of neutralizing antibody will provide protection, the ability to show cross-neutralization against emerging variants indicates cross-protection. A total of 244 serum samples were collected from 100 individuals between March 2021 and March 2022, ranging between 12 days to 976 days post-symptom onset. Results showed that neutralizing antibody levels remained relatively unchanged against the Ancestral strain and the Delta variant for the first six months, declining significantly after, despite vaccination. In contrast, neutralizing antibodies increased with time against the Omicron variant, but this may be because samples were collected when the Omicron variant was still prevalent. A small number of individuals reported reinfection, 9/100. However, more could have been asymptomatically infected, which could account for the observed increase in the titer of neutralizing antibodies against the Omicron variant. Results also showed that vaccinated individuals had significantly higher neutralizing antibodies compared to unvaccinated, irrespective of which variant was responsible for initial infection. Serum samples were collected from 62 individuals in the final year of the study. Among the 62, 69.4% (43/62), 64.5% (40/62), and 69.4% (43/62) with neutralizing antibodies against the Ancestral strain, Delta, and Omicron variants were vaccinated against SARS-CoV-2, highlighting the effectiveness of vaccination and the importance of continued vaccination globally. A total of 85.4% (53/62), 80.6% (50/62), and 90.3% (56/62) still had detectable neutralizing antibodies against the Ancestral strain, Delta, and Omicron variants at the end of the study. Results confirmed cross-neutralization between variants, showing that vaccines based on Ancestral strain play an important role in inducing cross-neutralizing antibodies, even against heterologous variants. Most studies have reported waning immunity against SARS-CoV-2 based on detecting neutralizing antibodies (Chia et al., 2021; Goldberg et al., 2022). Others have reported that immunity remains stable and persists for months before waining (Haveri et al., 2021; Mai et al., 2022). According to our knowledge, this is the first study to report on immunity over two years post-infection, showing that SARS-CoV-2 IgG and neutralizing antibodies remain relatively stable for up to six months post-symptom onset before declining significantly, with most individuals still having detectable antibodies at two years post-symptom onset, showing longevity of antibodies. The main limitation was the inability to determine if the longevity was enhanced by reinfection, as this can be asymptomatic in many instances. Another limitation was

that patient demographics were not recorded at the beginning of the study, including age, comorbidities, the severity of infection, and whether individuals were hospitalized. Previous studies have shown that these factors play a major role in determining the strength of neutralizing antibodies in individuals. There are new variants currently circulating, including Eris, Pi, and Pirola, and the potential exists for further variants to emerge. The pi (BA.6) is another Omicron variant detected in late July 2023. Eris (EG.5.1) is a descendant of the Omicron variant, with clinical representation similar to other Omicron subvariants (Parums, 2023). The key difference between Eris and Omicron is a single mutation (F456L) compared to the Omicron subvariant XBB.1.5 (Veltri et al., 2023). Pirola (BA.2.8.6) has more than 30 new mutations and has been detected in Denmark, South Africa, Israel, the United States of America, and the United Kingdom (Meo et al., 2023). This shows that although the pandemic has been declared over, continued surveillance is important to track the virus and individual immunity to assess if individuals are still protected.

Previous studies have shown that vaccine-induced immunity can protect against COVID-19 and plays a role in controlling the spread of the virus. However, new immune-evading variants continue to emerge. Additionally, not all individuals are willing to vaccinate due to vaccine hesitancy. Thus, it is important to address vaccine hesitancy and educate individuals on the importance of vaccines, the value of protection they offer, and the benefits to a community. The results of this study show that there is longevity of immunity to SARS-CoV-2, which is likely boosted by vaccination or reinfection. The study also indicated that vaccination significantly boosts IgG and neutralizing antibodies, with vaccinated individuals developing stronger IgG and neutralizing antibodies compared to unvaccinated. Only a small proportion of individuals in the study reported reinfection. Although more may have potentially been infected with asymptomatic SARS-CoV-2, the low number of reinfections also shows that the persistence of antibodies may have reduced the chances of reinfection. Although immunity is not long-lasting, continued booster vaccination may be essential in maintaining immunity and protecting from possible reinfection. Thus, new vaccines that include new SARS-CoV-2 variants should be developed, as they would provide better protection as SARS-CoV-2 continues evolving.

The WHO has reported that 70.6% of the population has received at least a single dose of SARS-CoV-2 vaccine, 32.8% being from low-income countries

(https://ourworldindata.org/covid-vaccination). One study estimates that herd immunity required is about 60-72%, with an R_0 of 2.5-3.5, and if vaccine efficacy is considered to be 80% (ε is 0.8), then herd immunity becomes 75-90% (Anderson et al., 2020; D'Arienza and Coniglio, 2020). But due to waning immunity, even if herd immunity is reached, it will not eliminate the virus due to its mutating nature, zoonotic nature, and waning immunity. Thus, booster vaccinations are essential. Although they may not provide life-long protection, they can boost immunity and reduce the chances of reinfection or severity of illness.

Currently, approved vaccines have been shown to reduce disease severity and lower hospitalization and fatalities. However, treatment is still required for the management of the disease, and because of continued mutations, newer variants may partially or fully escape protection from the vaccines. Infection with SARS-CoV-2 can result in an uncontrolled release of proinflammatory cytokines, resulting in a cytokine storm, which plays a role in disease severity. IL-6, IL-1β, and TNF-α have been identified as the main proinflammatory cytokines associated with disease severity (Jing et al., 2022; Ashrafzadeh-Kian et al., 2022), and IL-6 is used as a disease severity biomarker (Chen et al., 2020). Studies have shown the effectiveness of plant extracts against different viral infections, including SARS-CoV-2 (Abd-Alla et al., 2019; Mehrbod et al., 2019; Thabti et al., 2020). Thus, research that focuses on novel treatments for COVID-19 should be underway. The study also aimed to determine the effects of immune modulators on innate immunity against SARS-CoV-2 Omicron variant. Phela, a plant extract under development at the Indigenous Knowledge Sytems Unit, University of the Free State, as an immune booster in South Africa, was investigated for activity against the virus *in vitro*. Phela and its components did not appear to reduce virus replication. However, the results suggest modulation of proinflammatory cytokine release. Treatment with plant extracts resulted in significantly lower release of IL-1β, IL-2Rα, and TNF-α, with better response post-treatment than pretreatment, showing that the plant extracts may have the potential to manage cytokine storms. However, further investigations must be performed to determine if similar results can be obtained in a clinical setting. The exact mechanism of action of these plant extracts still needs to be investigated to determine how they reduce pro-inflammation with the potential of being a source of treatment for not only SARS-CoV-2 but also other viruses in which infection results in the dysregulated proinflammatory immune responses. The

limitation of the study is that the small design and sample number warrant repeating the experiments with more samples and additional parameters and using gene expression studies or microarrays to further characterize the effect on the upregulation or downregulation of cytokines.

There are still some unknowns regarding COVID-19, which have significant effects on health, such as long-COVID, which affects individuals of all ages and disease severity and may persist for weeks to months. Individuals infected with long COVID present with various cognitive and autonomic dysfunctions. Several studies have been published, but the exact pathology behind these symptoms of SARS-CoV-2 is still unknown. Thus, more studies should be performed to investigate the connection between immunity and long COVID. A recent study showed increased circulation of antibodies against other viruses, including Epstein Barr, in individuals with long COVID compared to recovered individuals (Gehlhausen et al., 2023). Cortisol levels were also lower in individuals with long COVID (Gehlhausen et al., 2023). Therefore, continued research is important to clarify the pathology and long-term effects of COVID-19. It is crucial to continue virus surveillance, track emerging variants, monitor individual immunity, and administer vaccinations to boost waning immunity, especially since the current reported infection data does not reflect the actual infection rate due to reduced testing worldwide. Vaccine development against emerging variants could be important for sustained protection.

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Appendices

Appendix 1: Letter of approval from the Health Sciences Research Ethics Committee

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Health Sciences Research Ethics Committee

12-Aug-2021

Dear **Miss Matefo Litabe**

Ethics Number: UFS-HSD2020/2001/2601-0003

Ethics Clearance: **Adaptive immune response in COVID-19 patients and innate immune modulation of SARS-COV-2**

Principal Investigator: **Miss Matefo Litabe**

Department: **School of Pathology Department (Bloemfontein Campus)**

Submission Page

SUBSEQUENT SUBMISSION APPROVED

With reference to your recent submission for ethical clearance from the Health Sciences Research Ethics Committee. I am pleased to inform you on behalf of the HSREC that you have been granted ethical clearance for your request as stipulated below:

Approval is requested to collect samples for the study from members of the staff at the University of the Free State.
Dr Jansen and Riana Johnson from Kovsie Health have been approached to assist with collection of the samples.
An advert will be sent out requesting interested staff to contact the investigators to explain the research and possible enrolment, copy of the advert is attached.
Approval is requested to advise participants of the antibody results.
Participants will be advised that the test results are not diagnostic but are research. The tests used are not SAPHRA approved for diagnosis.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; International Council for Harmonisation (ICH) Harmonised Guideline, Integrated Addendum to ICH E6(R1), Guideline for Good Clinical Practice (GCP) E6(R2), 2016, SAHPRA Guidelines as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this request for ethical clearance and we wish you continued success with your research.

Yours Sincerely

Prof. A. Sherriff
Chairperson : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00011992; REC 230408-011; IORG 0010096; FWA 00027947
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

Appendix 2: Letter of Approval to Collect Samples for the University of the Free State Staff Members

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA
UFS UV

Office of the Vice-Rector: Research and Internationalisation
Kantoor van die Viserektor: Navorsing en Internasionalisering

02-Aug-2021

Dear Miss Matefo Litabe

UFS AUTHORITIES APPROVAL

Research Project Title:
Adaptive immune response in COVID-19 patients and innate immune modulation of SARS-COV-2

This letter serves as confirmation that your request to collect data from students and/or staff members at the University of the Free State for your research project has been approved provided that you also have ethical clearance for the research from the ethics committee at the University of the Free State.

Please make sure that you also obtain your ethics clearance letter containing your reference number from the ethics committee after you have received this letter before you conduct your research.

Kind Regards

PROF RC WITTHUHN
VICE-RECTOR: RESEARCH & INTERNATIONALISATION
CHAIR: SENATE RESEARCH ETHICS COMMITTEE

205 Nelson Mandela Drive/Rylaan
Park West/Parkwes
Bloemfontein 9301
South Africa/Suid-Afrika

P.O. Box / Posbus 339
Bloemfontein 9300
South Africa / Suid-Afrika
T +27(0)51 401 2116
F +27(0)51 401 3762
WitthuhnRC@ufs.ac.za
www.ufs.ac.za

Appendix 3: Biosafety and Environmental Research Ethics Committee Approval Letter

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
RESEARCH DEVELOPMENT
NAVORSINGSONTWIKKELING

Environment & Biosafety Research Ethics Committee

10-May-2021

Dear Miss Matefo Litabe

Project Title: Adaptive immune response in COVID-19 patients and innate immune modulation of SARS-CoV-2

Department: Medical Microbiology Department (Bloemfontein Campus)

APPLICATION APPROVED

This letter confirms that this research proposal was given ethical clearance by the Environment & Biosafety Research Ethics Committee of the University of the Free State.

Your ethical clearance number, to be used in all correspondence is: **UFS-ESD2020/0181**

Please note the following:

1. **This ethical clearance is granted for data collection for this project for the duration of 03 Year/s from the issuance of this letter.**
2. **If the duration of the data colection indicated above is less than one year, your ethics clearance will be valid for 12 months from the issuance of this letter. Please submit a Continuation Report to the Ethics Committee if your data collection takes longer than a year, or longer than the alloted time indicated above if it is longer than one year .**
3. **Please note that the maximum amount of time that ethical clearance will be granted is 5 years, and that a Continuation Report must be submitted for any projects where data is collected for longer.**
4. **If any changes are made during the research process (including a change in investigators), please inform the Ethics Committee by submitting an Amendment.**
5. **When the research is concluded, please submit a Final Report to the Ethics Committee.**

Thank you for your application and we wish you well in all of your research endeavours.

Yours Sincerely

Prof. RR (Robert) Bragg
Chairperson: Environment & Biosafety Research Ethics Committee
University of the Free State

Appendix 4: Section 20 from the Department of Agriculture, Land Reform, and Rural Development

agriculture, land reform & rural development

Department
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development
Private Bag X250, Pretoria 0001

Enquiries: Mr Henry Gololo · Tel: 32· Fax: +27 12 319 7470 E-mail: HenryG@daff.nrl.gov.za
Reference: 12/11/1/4 (1629 LH)

Prof Felicity Burt
University of the Free State, Division of Virology
205 Nelson Mandela Drive
Bloemfontein

Dear Prof Burt,

Re: Section 20 Application Titled "Neutralising antibody assay for validation of assays and monitoring immune responses in COVID-19 patients"

Your Section 20 application that was submitted on 27 August 2020 refers.

Based on the information provided, you do not need Section 20 approval in terms of the Animal Diseases Act, 1984 (Act No 35 of 1984) subject to compliance with the following conditions:

1. Obtain a valid veterinary import permit for the importation of the vero cells prior to the importation thereof;
2. Obtain written permission from National Department of Health to culture, handle, manipulate etc. SARS-CoV-2 in the proposed BSL3 laboratory.

For point 2 above you can contact lineo.motopi@health.gov.za

Kind regards,

Maja.

Dr Mpho Maja
DIRECTOR: ANIMAL HEALTH
Date: 2020 -09- 01

cc: lineo.motopi@health.gov.za

– 1 –

Appendix 5: Consent form for collection of samples in three languages

CONSENT DOCUMENT 1 Version 2 (29 March 2020) ECUFS NR……..

CONSENT TO PARTICIPATE IN RESEARCH

PROJECT TITLE: Determining antibody responses to severe acute respiratory syndrome coronavirus 2

You have been asked to participate in a research study.

You have been informed about the study by …….

You may contact Prof FJ Burt at 051 401 3461 any time if you have questions about the research. You may contact the Secretariat of the Ethics Committee of the Faculty of Health Sciences, UFS at telephone number (051) 4017794/5 if you have questions about your rights as a research subject.

Your participation in this research is voluntary, and you will not be penalized if you refuse to participate or decide to terminate participation. If you agree to participate, you will be given a signed copy of this document as well as the participant information sheet, which is a written summary of the research.

The research study, including the above information has been verbally described to me. I understand what my involvement in the study means and I voluntarily agree to participate. I consent that

1. I will donate blood and answer the questions on this form related to onset of illness and symptoms of diseases
2. My donated blood may be stored and analysed as part of future research
3. Research data obtained from the study may be published anonymously in a scientific journal

Name of participant **Telephone no**

Email address

_____ _____

Signature of Participant Date

_____ _____

Signature of Witness Date
(Where applicable)

_____ _____

Signature of Translator
(Where applicable)

Date

Information

Please complete the following questions:

When did you test positive for COVID-19? ……

When did your initial symptoms begin? .....

Follow up questions

Have you tested positive again for COVID-19? .....

If yes, when? .....

Have you received a booster vaccine? ....

If yes, what date did you receive the vaccine? .....

Follow up questions

Have you tested positive again for COVID-19? .....

If yes, when? .....

Have you received a booster vaccine? ....

If yes, what date did you receive the vaccine? .....

INFORMATION DOCUMENT 1 Version 2 (29 March 2020) ECUFS NR……..

INFORMATION DOCUMENT

PROJECT TITLE: Determining antibody responses to severe acute respiratory syndrome coronavirus 2

Good day,

We, the Division of Virology, are doing research to investigate how long it takes for your body to produce antibodies against the new coronavirus and how long these antibodies are present in the body. Antibodies are proteins in your blood that help to protect your body against infections. We also want to determine how long the antibodies are present in your blood which is why we need to contact you one and two years after onset of illness. It is useful to scientists to understand this response as it contributes to our knowledge and is used for identifying possible treatment.

Invitation to participate - We are inviting you to participate in a research study.

What is involved in the study – We are inviting people that have tested positive for SARS-CoV-2 virus to take part in a three-year study to determine how long antibodies are present after the onset of illness. For each participant, we will request blood samples, as described below.
1. We will request one x 10 ml of blood during the initial year after infection.
2. We will request one x 10 ml blood sample from you at one year and two years after the onset of illness.

In the laboratory, we will test the blood for antibodies against the virus.

We request permission to store your blood in the Division of Virology for use in future studies related to SARS-CoV-2 or other zoonotic pathogens as control samples in the development or validation of laboratory tests.

Risks - There are no foreseeable risks of being involved in the study. There might be momentary discomfort at the site of the blood draw, possible bruising, redness, and swelling around the site of the blood draw. The blood draw will be done by a trained medical doctor to minimize the risks.

Benefits - There is very little information on when antibodies are produced by the body against the virus and which tests are useful for the diagnosis of the disease. This information will help in the knowledge and understanding of how the body responds to this virus.

Participation - Participation is voluntary, and refusal to participate will involve no penalty; the patient may discontinue participation at any time.

Reimbursements and remunerations - All procedures will be performed at no extra cost to you. You will not receive any remuneration for participating in the study.

Cost - Participant will not pay any costs to participate in the study

Confidentiality - Efforts will be made to keep personal information confidential. Absolute confidentiality cannot be guaranteed. Personal information may be disclosed if required by law. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as the Ethics Committee for Medical Research. If results are published, this may lead to individual/cohort identification.

Contact details of researcher(s) – Matefo Litabe, Division of Virology, University of the Free State, Bloemfontein. Tel 051 405 3082 millicentlitabe@gmail.com
Supervisor: Prof Felicity Burt, Division of Virology, University of the Free State, Bloemfontein. Tel 051 401 3461 burtfj@ufs.ac.za

Contact details of Secretariat and Chair: Ethics Committee of the Faculty of Health Sciences, University of the Free State – for reporting of complaints/problems: Telephone number (051) 4017794/5 EthicsFHS@ufs.ac.za

TOESTEMMINGS DOKUMENT 1 Weergawe 2 (Oktober 2020) HSREC NR

TOESTEMMING VIR DEELNAME AAN NAVORSING

PROJEK TITEL: Die bepaling van die teenliggaamsreaksies teen akute asemhalingsindroom Coronavirus 2 (SARS-CoV-2)

U word versoek om aan ’n navorsingstudie deel te neem.

U is oor die studie ingelig deur……..

U kan enige tyd vir Prof Felicity Burt kontak by 051 401 3461 indien u vrae oor die navorsingstudie het. U kan die Sekretariaat van die Etiekkomitee van die Fakulteit Gesondheidswetenskappe, UV by telefoonnommer (051) 401 7794/5 kontak indien u enige vrae oor jou regte as 'n navorsingsdeelnemer het.

U deelname aan hierdie navorsingstudie is vrywillig en u sal nie benadeel word of enige voordele verloor indien u weier om aan die studie deel te neem of besluit om deelname te beëindig nie. Indien u instem om deel te neem, sal u 'n getekende afskrif van hierdie dokument, asook die deelnemer inligtingsdokument, wat 'n geskrewe opsomming van die navorsingstudie is, gegee word.

Die navorsingstudie, insluitend die bogenoemde inligting is verbaal aan my beskryf. Ek begryp wat my betrokkenheid by die studie beteken en ek stem vrywilliglik in om deel te neem.

Ek gee toestemming dat:

1. Ek bloed sal skenk, soos beskryf in die inligtingsdokument en die vrae op hierdie vorm beantwoord aangaande die aanvang van siekte en die simptome daarvan.
2. My geskenkte bloed gestoor en geanaliseer mag word as deel van toekomstige navorsing
3. Navorsingsdata wat verkry word van die studie mag anoniem gepubliseer word in 'n wetenskaplike joernaal.

Naam van deelnemer **Telefoon nr**

_____

E-pos adres

_____ _____

Handtekening van deelnemer Datum

_____ _____

Handtekening van getuie Datum
(Waar van toepassing)

Inligting

Voltooi asb op die dag van werwing

Wanneer het u positief getoets vir COVID-19? .....

Wanneer het die eerste simptome begin? .....

INLIGTINGS DOKUMENT 1 Weergawe 2 (Oktober 2020) HSREC NR .....

INLIGTINGSDOKUMENT

STUDIE TITEL: Die bepaling van die teenliggaamsreaksies teen akute asemhalingsindroom Coronavirus 2 (SARS-CoV-2)

Goeie dag

Die divisie van Virologie aan die Universiteit van die Vrystaat, doen navorsing om te ondersoek hoe lank dit neem om teenliggame teen die nuwe coronavirus te produseer. Teenliggame is proteïne in u bloed wat u beskerm teen infeksies. ons wil ook bepaal hoe lank die teenliggaampies in u bloed voorkom, daarom moet ons een en twee jaar na infeksie met u in verbinding tree. Dit is vir ons as wetenskaplikes baie nuttig om hierdie interaksie te verstaan, want dit dra by tot die ontwikkeling van moontlike geneesmiddels.

Uitnodiging om deel te neem: Ons nooi u om deel te neem aan 'n navorsingstudie.

Wat behels die studie: Ons nooi mense uit wat positief getoets is vir SARS-CoV-2 om aan 'n drie jare studie deel te neem. Die studie sal bepaal hoe lank teenliggaampies teenwoordig is ná die aanvang van die siekte. Ons sal bloedmonsters aanvra soos hieronder beskryf:

1. Ons vra vyf 10 ml bloed gedurende die eerste jaar van infeksie
2. Ons vra vyf 10 ml bloed die eerste en tweede jaar na infeksie

Ons sal die bloed toets vir teenliggame teen die virus in die Divisie van Virologie se laboratoriums.

Ons versoek toestemming van u om u bloed te stoor in die Divisie van Virologie vir gebruik in toekomstige studies oor SARS-CoV-2 of ander soönotiese patogene as kontrolemonsters vir die ontwikkeling of validering van laboratoriumtoetse.

Risiko: Daar is geen voorsienbare risiko met u betrokkenheid in die studie nie. Daar mag moontlik tydelike ongemak by die area waar die bloed getrek word voorkom, soos kneusplekke, rooierige voorkoms en swelling. Bloed sal getrek word deur ‘n opgeleide mediese dokter om die betrokke risiko’s te beperk.

Voordele: Daar sal geen direkte voordeel uit u deelname aan die studie wees nie. Daar is min inligting beskikbaar oor wanneer teenliggame teen die virus deur die liggaam geproduseer word en watter diagnostiese toetse die mees bruikbaar is. Die inligting wat uit die studie verkry word, sal help om te verstaan hoe die liggaam reageer teen die virus.

Deelname: Deelname is vrywillig, en weiering om deel te neem sal geen boete behels nie; u kan ter enige tyd aan deelname onttrek.

Vergoeding: Alle prosedures sal uitgevoer word sonder dat enige onkostes van u verhaal word. U sal geen vergoeding ontvang vir u deelname aan die studie nie.

Koste: U sal geen onkostes hê vir deelname aan die studie nie.

Vertroulikheid: Daar sal gepoog word om persoonlike inligting vertroulik te hou. Volkome vertroulikheid kan nie gewaarborg word nie. Persoonlike inligting kan bekend gemaak word as die wet dit vereis. Organisasies wat u navorsingsrekords mag ondersoek en/of kopieer vir kwaliteitsversekering en data-analise sluit groepe soos die Etiekkomitee vir Mediese Navorsing in.

Kontakbesonderhede van navorser(s): Matefo Litabe, Divisie van Virologie, Universiteit van die Vrystaat, Bloemfontein. Tel 051 405 3082 millicentlitabe@gmail.com
Toesighouer: Prof Felicity Burt, Divisie van Virologie, Universiteit van die Vrystaat, Bloemfontein. Tel 051 401 3461, burtfj@ufs.ac.za

Kontakbesonderhede van sekretariaat en Voorsitter: Etiekkomitee van die Fakulteit Gesondheidswetenskappe, Universiteit van die Vrystaat - vir rapportering van klagtes of probleme: Telefoonnommer (051) 401 7794/5 EthicsFHS@ufs.ac.za

TOKOMANE YA TUMELO YA 1 Kgatiso ya 2 (21 Mmesa 2020) HSREC NR……..

TUMELO YA HO NKA KAROLO PATLISISONG

SEHLOOHO SA PROJEKTE: Ho hlahloba sesole sa mmele kgahlanong le *respiratory syndrome coronavirus 2* e totileng

O kopilwe ho nka karolo phuputsong ya boithuto.

O tsebisitswe mabapi le phuputso ke …… .

O ka ikopanya le Prof FJ Burt ho 051 401 3461 ka nako efe kapa efe haeba o na le dipotso mabapi le patlisiso. O ka nna wa ikopanya le Bongodi ba Komiti ya Melawana ya Boitshwaro Tshebetsong ya Lefapha la Disaense tsa Bophelo, ya UFS nomorong ya mohala ya (051) 4017794/ 4017794/5 haeba o na le dipotso mabapi le ditokelo tsa hao jwalo ka mohlahlobuwa patlisisong.

Ho nka karolo ha hao patlisisong ena ho etswa ka boithaopo, mme o ke ke wa fuwa kotlo haeba o hana ho nka karolo kapa o etsa qeto ya ho fedisa bonkakarolo. Haeba o dumela ho nka karolo, o tla fuwa khopi e saennweng ya tokomane ena mmoho le pampitshana ya tlhahisoleseding ya monkakarolo, e leng kakaretso ya mongolo ya patlisiso.

Phuputso ya boithuto, e kenyeletsang tlhahisoleseding e boletsweng ka hodimo mona ke e hlaloseditswe ka puo. Ke utlwisisa hore se bolelwang ke kameho ya ka phuputsong ena ke hore ke dumela ka boithaopo ho nka karolo. Ke dumela hore:

1. Ke tla fana ka madi le ho araba dipotso tse foromong ena tse amanang le qaleho ya bokudi le matshwao a ho kula a mafu
2. Madi a ka ao ke faneng ka wona a tla bolokwa le ho manollwa e le karolo ya patlisiso ya kamoso
3. Lesedi la patlisiso le fumanweng ho tswa phuputsong le ka nna la patlalatswa ka tsela e sa bontsheng lebitso jenaleng ya tsa saense

Lebitso la monkakarolo **Nmr. ya mohala**

Aterese ya imeile

_____ _____

Tshaeno ya Monkakarolo Letsatsi

_____ _____

Tshaeno ya Paki Letsatsi
(Moo ho hlokehang)

Tlhahisoleseding

Ka kopo tlatsa letsatsing la ngodiso

Liteko tsa hao tsa COVID-19 li fumanoe li le positive neng? .....

Matsoao o hao a pele a ho kula a qadile neng? .....

TOKOMANE YA TLHAHISOLESEDING YA 1 Kgatiso ya 1 (21 Mmesa 2020)
HSREC NR……..

TOKOMANE YA TLHAHISOLESEDING

SEHLOOHO SA PROJEKTE : Ho hlahloba sesole sa mmele kgahlanong le *respiratory syndrome coronavirus 2* e totileng

Dumela
Rona, Lekala la Viroloji, re etsa patlisiso e fuputsang hore ho nka nako e kae hore mmele o hlahise masole a mmele kgahlanong le coronavirus e ncha, le hore na a phehella nako e kae. Masole a mmele ke diprotheine tse mading a hao tse thusang ho sireletsa mmele wa hao kgahlanong le ditshwaetso. Re batla ho tseba hore na masole ana a mmele kgahlanong le coronavirus e ncha a fumaneha mading a hao halelele ha kae. Ke ka hona re hlokang ho ikopanya le wena selemong sa pele le sa bobedi ka mora tswaetso. Ho molemo hore borasaense ba utlwisise hore karabelo ena e tlatsetsa tsebong ya rona hobane e sebedisetswa ho tsebahatsa kalafo e ka kgonehang.

Memo ya ho nka karolo: Re o memela ho nka karolo phuputsong ena ya boithuto.

Ke eng se amehang phuputsong ee – Re mema batho ba hlahobileng ba na le tsoaetso ya COVID-19 ho nka karolo dipatlisisong tsa dilemo tse tharo. Re tla kopa disampole tsa madi tse latelang:

1. Re kopa 10ml e le 'ngoe ea mali selemong sa pele sa tsoaetso
2. Re kopa 10ml e le 'ngoe ea mali ka selemo sa pele le sa bobedi kamora tsoaetso

Laboratoring re tla etsa teko ya madi bakeng sa masole a mmele a sebetsang kgahlanong le vaerase

Re kopa tumello ea ho boloka mali a hao Lekaleng la Virology bakeng sa lithuto tsa kamoso tse amanang le SARS-CoV-2 kapa likokoana-hloko tse ling e le lisampole tsa taolo ntshetsopele kapa netefatso ea liteko tsa laboratori.

Dikotsi Ha ho dikotsi tse lebeletsweng tsa ho ameha phuputsong ena.

Melemo Ho na le tlhahisoleseding e nyane haholo mabapi le hore na masole a mmele a hlahiswa neng ke mmele kgahlanong le vaerase le hore ke diteko dife tse molemo bakeng sa phumano ya lefu. Tlhahisoleseding ena e tla thusa tsebong le kutlwisisong ya ka moo mmele o arabelang vaerase ena.

Bonkakarolo ke ba boithaopo, mme ho hana ho nka karolo ho ke ke ha baka kotlo ya letho; mohlahlobuwa a ka nna a kgaotsa ho nka karolo ka nako efe kapa efe.

Dipuseletso tsa tjhelete Mekgwatshebetso - yohle e tla etswa ntle le tjeo ho wena. O ke ke wa fumana dipuseletso tsa tjhelete efe kapa efe bakeng sa ho nka karolo phuputsong ena.
Tjeo: Monkakarolo a ke ke a lefa ditjeo tsa letho ho nka karolo phuputsong

Sephiri: Boikgathatso bo tla etswa ho boloka tlhahisoleseding ya hao sephiring. O ke ke wa fuwa tiisetso ya sephiri se feletseng ka hohlehohle. Tlhahisoleseding e mabapi le wena e ka hlahiswa haeba molao o hloka jwalo. Mekgatlo e ka hlahlobang le/kapa e kopitsang direkoto tsa hao tsa diphuputsong bakeng sa netefatso ya boleng le tekodiso ya data e kenyeletsa dihlopha tse jwalo ka Komiti ya Melawana ya Boitshwaro Tshebetsong bakeng sa Patlisiso ya Bongaka. Haeba diphetho di phatlalatswa, sena se ka nna sa lebisa tsebahatsong ya motho/sehlopha.

Dintlha tsa boikopanyo tsa (ba)mofuputsi – Matefo Litabe, Division of Virology, University of the Free State. Tel 051 405 3082 millicentlitabe@gmil.com
Mookamedi: Prof Felicity Burt, Division of Virology, University of the Free State, Bloemfontein. Mohala 051 401 3461, burtfj@ufs.ac.za

Dintlha tse mabapi le boikopanyo tsa Bongodi le Modulasetulo: Ethics Committee of the Faculty of Health Sciences, University of the Free State – bakeng sa ho tlaleha ditletlebo/mathata: Nomoro ya mohala (051) 4017794/5 EthicsFHS@ufs.ac.za