Evaluation of Two SARS-CoV-2 Immunoassays

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ABSTRACT

Aim: The purpose of this study is to verify the performance of the Roche Elecsys ® anti-nucleocapsid (qualitative) and anti-spike (quantitative) SARS-CoV-2 immunoassays to determine whether the performance of the assays is acceptable for diagnostic use in the Groote Schuur Hospital virology/chemistry laboratory, as well as other National Health Laboratory Service (NHLS) laboratories in South Africa.

Methods: We performed a verification study using de-identified remnant serum or plasma samples. Standard verification experiments including sensitivity, specificity and precision were performed. Pre-pandemic samples were used to assess specificity. Samples with a linked positive SARS-CoV-2 polymerase chain reaction (PCR) result on a respiratory sample >10 days before the serum/plasma collection date were used to assess sensitivity. Additionally, post-vaccine humoral response and other parameters was assessed in a cohort of laboratory staff.

Results: For the anti-nucleocapsid antibody assay, specificity was 99.7% based on 316 samples and sensitivity 91.3% based on 404 samples. For the anti-spike antibody assay, the specificity based on 194 samples was 100%, and the sensitivity based on 384 samples was 93.8%. Both assays demonstrated acceptable precision. Furthermore, the anti-spike antibody assay sensitivity was >92% during the first three waves in South Africa, dominated by different SARS-CoV-2 variants. Post-vaccine seroconversion in 115 staff with no evidence of prior natural infection was 99% and hybrid immunity produced higher anti-spike antibody titres compared to vaccine-only participants.

Conclusion: Both immunoassays met our acceptance criteria. Both assays can be used for seroprevalence studies. The anti-nucleocapsid immunoassay assay is valuable in confirming past natural infection in patients with previous asymptomatic infection, previous symptomatic infection where no PCR was done or PCR-negative patients who present to hospital with COVID-19 during the second week of illness or later. Most importantly, the anti-spike immunoassay can be used as a reliable, cheap, and easily accessible surrogate marker of post-vaccine humoral immune response and we recommend using this to confirm and monitor humoral immune response in patients with risk factors for non-seroconversion following vaccination and increased risk for morbidity and mortality following infection with SARS-CoV-2.

DECLARATION

The research reported is based on independent work performed by the candidate and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree to any other university. This work has not been reported or published prior to registration for the abovementioned degree.

This thesis/dissertation has been submitted to the Turnitin module (or equivalent similarity and originality checking software) and I confirm that my supervisor has seen my report and any concerns revealed by such have been resolved with my supervisor.

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ABBREVIATIONS

95%CI: 95% confidence interval

ACE-2: Angiotensin-converting enzyme 2

ADCC: Antibody-dependent cellular cytoxicity

CLIA: Chemiluminescent assay

CLSI: Clinical and Laboratory Standards Institute

CMV: Cytomegalovirus

%CV: Co-efficient of variation percentage

COVID-19: Coronavirus disease 2019

Ct: Cycle threshold

EBV: Epstein-Barr virus

E: Envelope protein

ELISA: Enzyme-linked immunosorbent assay

FIND: Foundation for Innovative New Diagnostics

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IQC: Internal quality control

IQR: Interquartile range

M: Membrane protein

MERS-CoV: Middle East respiratory syndrome coronavirus

mRNA: Messenger ribonucleic acid

NHLS: National Health Laboratory Service

N: Nucleocapsid protein

n: number

OD: Optical densities

PCR: Polymerase chain reaction

RBD: Receptor binding domain

RDT: Rapid immunodiagnostic test

SAHPRA: South African Health Products Regulatory Authority

S1: Spike subunit 1

S2: Spike subunit 2

SARS-CoV-1: Severe acute respiratory syndrome coronavirus 1

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

S/CO: Signal/cut-off

S: Spike protein

SD: Standard deviation

VNA: Viral neutralization assay

WHO: World Health Organization

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CHAPTER 1

1.1 LITERATURE REVIEW

1.1.1 Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel coronavirus and the causative pathogen of coronavirus infectious disease 2019 (COVID-19) (1). There is still a paucity of clear knowledge concerning elicited humoral immune responses against SARS-CoV-2, particularly understanding of the magnitude, durability, how responses differ following natural infection versus vaccination, and the effects of different dominant circulating viral variants and how this may affect laboratory diagnostics. This knowledge is essential to help predict the trajectory of this pandemic and guide public health strategies and the judicious use of vaccines and diagnostic tests.

SARS-CoV-2 belongs to the family Coronaviridae and the genus Betacoronavirus. Four other endemic, seasonal human coronaviruses also belong to this family and are the causative agents of the common cold, namely 229E, NL63, OC43 and HKU1. Severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) and Middle East respiratory syndrome coronavirus (MERS-CoV) are two other zoonotic coronaviruses that have crossed over from animals to humans but have not achieved endemicity. A review of humoral immune responses to the aforementioned viruses is crucial to understanding those immune responses elicited by SARS-CoV-2 (1-3).

The genomes of all coronaviruses within the Coronaviridae family code for 4 structural proteins: the spike (S) protein, envelope (E) protein, membrane (M) protein and nucleocapsid (N) protein. The S protein is composed of two subunits, namely S1 and S2. The S1 subunit contains the receptor binding domain (RBD) and plays a key role in recognition and viral attachment to the host angiotensin-converting enzyme 2 (ACE-2) receptor, while the S2 subunit facilitates viral entry into the host cell via membrane fusion. The spike RBD, which serves as the main immunological target for the production of neutralizing antibodies, has 73-76% sequence homology between SARS-CoV-2 and SARS-CoV-1 (4).

Ongoing transmission of SARS-CoV-2 has led to one or more evolutionary changes in the virus, giving rise to several variants. These variants may display characteristics such as increased rate of transmission, an association with more severe clinical disease, reduced effectiveness of public health measures, treatments and vaccines, decrease in diagnostic performance or immune escape leading to increased risk for reinfection. (1,2,3) South Africa reported its first PCR-confirmed case of SARS-CoV-2 in March 2020 and as of mid-2021, the country experienced three waves of infection with a different dominating variant each. (4) Wild-type virus was the dominant virus circulating in South Africa during the first wave which occurred from June to early September 2020, with a peak July. The second wave of infection was predominated by the beta variant (B.1.351) which was first detected in October 2020 in the Eastern Cape. This variant was associated with multiple mutations in the spike protein and increased transmissibility which resulted in a larger peak (observed in December 2020) than seen during the first wave. The third wave, predominated by the delta variant (B.1.617.2), occurred from June to September 2021, with a peak in July. This variant was first detected in India and was associated with RBD spike protein mutations which contributed to a further increase in viral transmissibility(5-10).

1.1.2 Serological characteristics of SARS-CoV-2

Following exposure, symptom onset occurs after an incubation period of 2-7 days (median of 4 days) (11). Seroconversion rates to viral structural proteins have largely been reported to be nearing 100% but may differ based mainly on timing of testing within the course of disease and the performance and antigenic target of the serological assay used, namely whether total antibody or a specific immunoglobulin class (IgM, IgA or IgG) is being detected. Time to seroconversion ranges between 7-28 days for total antibody, IgG, and IgM, with the median seroconversion at 13 days post-symptom onset and maximum seroconversion at 20 days post-symptom onset. Seroconversion profiles for IgM and IgG have been inconsistent and has been reported to be either simultaneous or sequential, with IgM detection followed by IgG (12-14). The presence of total antibodies against N protein indicates past SARS-CoV-2 infection. The S protein is the target antigen in all currently used SARS-CoV-2 vaccines and thus, in contrast to anti-N antibodies, total antibodies against S protein are produced in response to both natural infection and/or vaccination. However,

like with other serological tests, failure to seroconvert following infection or vaccination, waning humoral immunity, and a false positive or negative test should also be considered when interpreting such results (15).

1.1.3 Magnitude of humoral immune response

Factors proposed to influence the magnitude of humoral immune response to SARS-CoV-2 infection may be clinical factors including disease severity and co-morbidities, or demographic factors such age, sex, and ethnicity. Disease severity has thus far shown the greatest influence on the magnitude of antibody response, with higher titres reported in severe COVID-19 (often hospitalised patients requiring oxygen support). In contrast, outpatients with asymptomatic or mild disease have been shown to develop lower antibody titres and a more rapid decline in spike RBD-specific IgM and IgG (16). Data on age and sex as correlates of antibody response is varied and thus far inconclusive. Very limited data exists concerning the effect of ethnicity on the degree of induced humoral response (17). Binding antibody responses i.e., anti-spike and anti-nucleocapsid IgG responses in 514 healthcare workers was measured 21 days after a single dose of the BNT162b2 mRNA (Pfizer-BioNTech) COVID-19 vaccine and compared according to age, sex, ethnicity and prior SARS-CoV-2 infection. Immunogenicity decreased with age, had no difference for sex and ethnicity, and elicited higher antibody titres in those with prior infection (18).

1.1.4 Durability of humoral immune responses against SARS-CoV-2 and other coronaviruses

Humoral immune response studies in patients with severe MERS-CoV, defined as those with pneumonia requiring supplementary oxygen support and/or ventilation have shown that severe clinical disease positively correlated with higher neutralizing antibody titres and longevity in detectable antibodies, often 1-2 years after infection. In contrast, asymptomatic and mild MERS-CoV infections were associated with lower seroconversion rates or a rapid decline in neutralizing and anti-spike antibodies to an undetectable level by 1-year post-infection in those who did seroconvert (19, 20). These studies were limited by the small number of participants being followed up.

SARS-CoV-1, the virus responsible for the 2002-2004 pandemic and the closest genetic relation to SARS-CoV-2, was mainly associated with symptomatic infection and asymptomatic and mild infection was infrequent. A longitudinal study evaluating humoral immunity in 176

convalescent SARS-CoV-1 patients showed 100% SARS-CoV-1 specific IgG positivity by 90 days post-infection, 94% at one year, 89.58% at 2 years and approximately 50% at 3 years post-infection. Optical densities (OD) of the commercial enzyme-linked immunosorbent assay (ELISA) used for this study correlated well with the IgG findings, with ODs peaking at 90 days post-infection but the rate of reduction in the average OD readings was much faster, declining by 22% at one year and 40% at two years post-infection (21). Another longitudinal study of 19 convalescent SARS-CoV-1 patients followed up at different time points until three years post infection, showed nucleocapsid antibody ODs drastically declined between three and 12 months and thereafter decreased more steadily until ODs were just above the cut-off after month 12 post infection. Additionally, at the 3-year mark, whole-virus specific antibody positivity was 42%, spike antibody positivity was 100% and neutralizing antibody activity was detectable in 89% of participants. Furthermore, anti-spike IgG titres positively correlated with neutralizing antibody activity (Spearman's correlation coefficient was 0.717) (22). A 2-year cohort study involving 56 convalescent SARS-CoV-1 patients showed that IgG and neutralizing antibody titres peaked at four months post infection and though at month 16, all patients still had detectable IgG, 11.8% were negative by the 2-year mark. As for neutralizing antibody titres, though positivity at the 2-year mark was 100%, titres dramatically declined from 16 months post infection onwards (23).

Data regarding antibody longevity is limited mainly by the fact that the current SARS-CoV-2 pandemic is less than three years in duration. Observational data from a national clinical laboratory in the United States of America showed 90% seropositivity for spike and nucleocapsid antibodies at 21 days post positive polymerase chain reaction (PCR) and at the 10-month mark nucleocapsid IgG antibody positivity was 68% and spike antibody positivity was 87.8%. It was also noted that individuals <65 years of age had higher rates of sustained seropositivity (24). A large longitudinal cross-sectional study in China demonstrated sustained seropositivity at eight months post positive PCR though neutralizing antibody titres did decline and were specifically lower in those who had asymptomatic infection (25). Similar results were demonstrated by other studies investigating antibody durability (26-28). However, data over longer time periods are still essential to assimilate our understanding of SARS-CoV-2 antibody durability and protection from re-infection.

1.1.5 Cross-reactivity with other coronaviruses

The spike proteins of other human beta coronaviruses (HKU1 and OC43 and MERS-CoV) have a sequence homology of approximately 30% with SARS-CoV-2 spike protein, whereas human alpha coronaviruses (229E and NL63) have nearly 24% sequence homology with the SARS-CoV-2 spike protein. By far, SARS-CoV-1, is the most closely related human coronavirus to SARS-CoV-2 with a spike protein sequence homology of 77%. Furthermore, other protein sequences of SARS-CoV-1 and SARS-CoV-2, namely nucleocapsid, envelope and membrane protein, show 91%, 96% and 91% similarity, respectively (29).

Given this high degree of similarity between SARS-CoV-2 and other human coronaviruses, humoral cross-reactivity would not be surprising. It has been postulated that the low prevalence of severe COVID-19 disease in children less than 10 years was due to the high amount of seasonal coronavirus antibodies cross-reacting with that of SARS-CoV-2, since it is thought that children suffer from common colds more frequently. However, a pre-pandemic study showed that by the age of five years, children and adults have comparable seropositivity and titres of seasonal coronavirus antibodies. Interestingly, children demonstrated a higher degree of cross-reactive antibodies to SARS-CoV-2 but their serum samples, including those with the highest S/RBD cross-reactivity, demonstrated no cross-neutralization (30).

Similar studies showed that cross-reacting antibodies were found to be most frequent and strong between SARS-CoV-1 and SARS-CoV-2 in both directions, however cross-neutralization, for both RBD and non-RBD regions, was a rare occurrence, and even when present, showed very weak neutralization. Therefore, if protection is offered by cross reactivity the mechanism is not via cross-neutralizing antibodies. Thus, the role of seasonal human coronaviruses in the pathogenesis of COVID-19 is still not clearly understood and large diverse pre-pandemic human cohorts are necessary to elucidate this enigma of pre-existing immunity. Furthermore, given the small number of people who have been infected with MERS-CoV and SARS-CoV-1, the overall global effect on the pandemic would be very limited (31, 32).

1.1.6 Serological testing for SARS-CoV-2

Having endured multiple waves of SAR-CoV-2 infection and successfully introduced vaccination worldwide, we now move into the next era of the current pandemic which

demands that our diagnostic laboratory repertoire also evolves. Though viral nucleic acid PCR remains the gold standard for diagnosis of acute SARS-CoV-2 infection, serological testing has carved its own niche as a diagnostic tool. Key usage includes the retrospective diagnosis of patients who did not have PCR testing done during the acute phase of illness and epidemiological studies looking at population susceptibility, vaccine coverage and response and likelihood of protection from re-infection. Humoral immune responses can be assessed by using either an antibody binding assay or a neutralizing antibody assay. There is currently a multitude of immunoassays available including ELISA, rapid immunodiagnostic tests (RDT) and chemiluminescent (CLIA) assays, all of which may target various viral antigens and antibody classes. The viral neutralization assay remains the gold standard for determining protective immunity, though multiple studies have demonstrated the use of spike RDB binding IgG antibodies as reliable correlates of neutralizing antibodies. Newer immunoassays available on automated platforms capable of high throughput testing and use in a biosafety level 2 laboratory, in contrast to the slower, more laborious viral neutralization assay which requires biosafety level 3 facilities. Due to their rapid development, swift validations (often involving small sample numbers) and lack of standardization, the performance of many of the currently available immunoassays show great diversity, with sensitivities and specificities ranging from 26-100% and 83-100%, respectively(33-35).

1.1.7 Conclusion

Though much knowledge regarding the development and evolution of the humoral immune response to SARS-CoV-2 has been gained, there is still essential information outstanding regarding durability and magnitude of protection following natural infection and/or a COVID-19 vaccine. This limitation is currently largely unavoidable due to the young age of the pandemic itself. With the vast number of serological assays rapidly being developed, it can be easy for laboratories, researchers, clinicians, and the public to get lost in the scientific hype, the pandemic fatigue, and the hope of freedom that “immunity passports” might bring. Before diagnostic laboratories invest in these instruments and reagents, before clinicians request these tests and most importantly, before clinical decisions and large-scale public health strategies are formed using these serological results, thorough validations, a well-defined purpose, and guidance surrounding interpretation of these immunoassays has never been more crucial.

1.2 AIM

The primary aim of this study was to verify the performance of the Roche Elecsys ® Anti-SARS-CoV-2 (qualitative anti-nucleocapsid) and Anti-SARS-CoV-2 S (quantitative anti-spike) immunoassays and check that the manufacturer's specifications were met and to determine whether the performance of the assay was acceptable for diagnostic use in the Groote Schuur Hospital virology and chemistry laboratories, as well as other National Health Laboratory Service (NHLS) laboratories in South Africa.

A secondary aim of the study was to elucidate information regarding post-vaccine humoral immune response, and timing, magnitude and durability of this humoral immune response following natural infection and/or vaccination.

CHAPTER 2

2. METHODS

2.1 Study design

This research project was primarily a verification study of two SARS-CoV-2 immunoassays. Standard verification experiments were performed including sensitivity, specificity and precision studies.

Included as part of the performance evaluation for the quantitative SARS-CoV-2 anti-spike immunoassay, principally post-vaccine response, was a subgroup of voluntary laboratory staff who had a single blood sample taken after completion of a questionnaire and informed consent.

2.2 Study site and period

The study was conducted at the virology and chemistry NHLS laboratories at Groote Schuur Hospital in Western Cape, South Africa. This study was conducted between July 2020 and August 2021.

2.3 Study samples/population

Study samples for sensitivity and specificity were sourced from de-identified, stored remnant serum or plasma samples left over after completion of all routine diagnostic testing (chemistry and virology). More recent samples were stored in the -4˚C fridge, whilst older samples were in the -20˚C freezer storage. These samples were collected and re-registered with a new episode number, specific to the verification study. This newly allocated episode number, test code and its results were not visible to healthcare workers outside of the NHLS. At least 150 samples were used to assess sensitivity and specificity for both assays. The sample size selected was guided by the South African Health Products Regulatory Authority (SAHPRA) and evaluation protocols provided by the NHLS (36) and World Health Organization (WHO) collaborative partner, Foundation for Innovative New Diagnostics (FIND) (37) .

2.3.1 Specificity

To assess specificity, residual virology serology historical samples with collection dates before November 2019 were tested. The expectation was that all samples would be negative as this pre-dated the first reported case of SARS-CoV-2 in December 2019. For the anti-nucleocapsid

and anti-spike SARS-CoV-2 immunoassays, 316 samples and 194 samples were evaluated, respectively. Included in each group of samples (n=40/316 and n=39/194) were residual virology samples with equivocal serology results. These were included to control for possible false reactivity due to chronic illnesses such as autoimmune diseases and malignancies.

2.3.2 Sensitivity

To assess assay sensitivity, the NHLS TrakCare database (laboratory information system) was searched to retrieve residual, serum or plasma samples of patients with a corresponding positive SARS-CoV-2 PCR result on a respiratory sample taken at least 10 days prior to the collection date of the sample, allowing for average time to seroconversion. Sensitivity at different time intervals in days since PCR positivity was also characterised. Since this selection process was based solely on extracted SARS-CoV-2 PCR results, the corresponding severity of COVID-19 symptoms associated with each antibody titre in the sensitivity studies is unknown. A total of 404 and 384 samples were used for the sensitivity studies of the anti-nucleocapsid and anti-spike SARS-CoV-2 immunoassays, respectively. The total of 384 samples used for the sensitivity studies of the anti-spike immunoassay included samples representative of each of the first 3 SARS-CoV-2 waves experienced in South Africa. Each wave being associated with a different dominant SARS-CoV-2 variant, with mutations included in their spike protein. Each wave was defined as serum/plasma sample of patients with a positive SARS-CoV-2 PCR from the time period as follows:

- Wave one/wild-type virus: May - August 2020 (n=123)
- Wave two/Beta variant: November - January 2021 (n=161)
- Wave three/Delta variant: June - September 2021 (n=100)

2.3.3 Precision

We assessed precision, both inter-run and intra-assay, of the qualitative anti-nucleocapsid immunoassay in accordance with The Clinical and Laboratory Standards Institute (CLSI) EP12 A2 for evaluation of qualitative assays (38) . Five replicates of five samples with varying values around the assay cut-off of 1 was tested daily for five consecutive days.

To assess precision of the quantitative anti-spike immunoassay, five replicates of a single positive and negative kit internal quality control (IQC) with assigned target values were run daily over five consecutive days in accordance with CLSI EP15 A3 (39) . Being a quantitative

assay, we further interrogated the inter-run variability by running the following sample groups daily for five consecutive days:

- Five samples with a value around the assay cut-off of 0.8U/ml
- Five known positive samples of varying positivity (high, mid-range and low)
- Five known negative samples

2.3.4 Agreement

During the spike immunoassay verification, samples from the specificity and wave one and wave two sensitivity studies with adequate volume were tested for both anti-spike and anti-nucleocapsid antibodies. Wave three samples were not used for this assessment since we do not have access to vaccination history for these samples. Sensitivity and specificity were calculated for each assay and using Stata (Version 17) agreement and kappa scores were calculated for each of the three sets of samples.

2.3.5 Post-vaccine humoral immune response

As part of the national initiative to vaccinate all health care workers, more than 300 NHLS laboratory staff at Groote Schuur Hospital had received a SARS-CoV-2 vaccine targeted against the spike protein. At the time of the blood draw for this study, majority received the single dose Johnson & Johnson (Ad26.COV2. S viral vector) vaccine and a minority received a single dose of the Pfizer-BioNTech (BNT162b2) mRNA (messenger ribonucleic acid) vaccine.

NHLS laboratory staff from different departments at Groote Schuur Hospital (virology, chemistry, haematology, microbiology, tissue immunology, genetics, and laboratory administration and support), regardless of designation, were invited to voluntarily participate in this branch of the study. Permission for the execution of this study was elicited from the laboratory business unit manager, after which an email was sent to all staff, informing them of the study, and inviting those interested to participate. Copies of a consent form for a single blood draw **(Appendix 1)** and a questionnaire **(Appendix 2)** were attached in this email for staff to read through and consider at their leisure. The questionnaire issued served to document basic demographics, COVID-19 vaccine history, and details around previous PCR positive natural infection. Contact details of the primary investigator and supervisor were provided in case of any enquiries. Interested participants were asked to submit their names

to their respective departmental mangers, from whom lists of interested staff were collected by the primary investigator.

Permission for use of NHLS employed phlebotomists was also granted. In keeping with current COVID-19 infection prevention protocols, to prevent overcrowding in the phlebotomy area, a specific day for blood collection was allocated to each department. Five participants at a time were counselled, informed consent taken by the primary investigator and thereafter a single blood sample collected by a phlebotomist from participants who were >28 days post-vaccine to evaluate seroconversion rates and to compare antibody titres between those vaccinees with a history of natural infection and those without.

Pre-registered labels based on the lists collected from department managers were used to label blood samples before storage in a - 4˚C fridge in the department of virology until testing began.

Laboratory staff were chosen to investigate this branch of the study due their large degree of expressed interest and for ease of accessibility for recruitment, clarification of queries, sample collection and follow up. Additionally, familiarity and competency of staff with laboratory terminology and procedures, also allowed for more efficient proceedings.

2.4 Laboratory procedures

2.4.1 Immunoassay

The immunoassays evaluated in this study were the Roche Elecsys ® Anti-SARS-CoV-2 S immunoassay (quantitative anti-spike antibody) and Roche Elecsys ® Anti-SARS-CoV-2 immunoassay (qualitative anti-nucleocapsid antibody). Both assays are based on electrochemiluminescent technology and a double-antigen sandwich format. The anti-nucleocapsid immunoassay utilizes recombinant protein representing the nucleocapsid antigen and the anti-spike immunoassay uses a recombinant protein representing the RBD of the spike antigen. Both immunoassays detect total antibodies to SARS-CoV-2 in human serum and plasma. The anti-spike immunoassay additionally quantifies antibody titres in assay-defined units. The cut-off for positivity on the analyser is >1.0 for the anti-nucleocapsid immunoassay and ≥0.8 U/ml for the anti-spike immunoassay (40, 41).

Groote Schuur Hospital's NHLS virology department was chosen by SAHPRA as the official site

for the verification process of these two immunoassays before they could be utilised nationally. As per standard procedure, Roche Diagnostics provided reagents, calibrators and controls for 1000 qualitative anti-nucleocapsid tests and 1400 quantitative anti-spike tests. Lot numbers and expiry dates for reagents, calibrators and controls used for this evaluation were documented. Kits were stored according to the manufacturer's specifications. After sharing of the verification protocol with Roche, no further involvement of the manufacturer occurred until after completion of official verification report.

2.4.2 Instrument

The instrument used for this study was the Roche Cobas e 601, module e4 analyser. Optimal timing for processing study samples was arranged with the department manager and operating medical technologists to prevent disruption of routine diagnostics and workflow.

2.4.3 Sample testing

Storage of study samples and run frequency of calibration and controls were done in accordance to the manufacturer's requirements. Calibration frequency was once per a new reagent lot number. A positive and negative quality control was run at least once every 24 hours before any samples were processed, once per a new reagent kit, and following each calibration. Controls and calibrations were checked to make sure they were within the specified acceptable range before sample testing could begin.

For the anti-spike assay, where test values are above the quantifiable limit (250 U/mL) the sample can be diluted using Roche's universal diluent (1:10 up to 1:100). After the analyser performs the dilution, the software accounts for the dilution when calculating the endpoint sample concentration. The dilution and the software calculation are both done automatically by the analyser. A test is used for each dilution.

2.5 Statistical analysis

The data collected was stored in Microsoft Excel. The 95% confidence intervals (95%CI) were calculated for the specificity, sensitivity and post-vaccine immune response. The mean, standard deviation and co-efficient of variation percentage (% CV) were calculated for the precision studies using Microsoft Excel. Median [interquartile range (IQR)] were calculated, where appropriate, using Microsoft Excel. Stata (version 17) was used to calculate agreement

and kappa scores. The statistical analysis mentioned below was done using Prism GraphPad (version 8). All data produced from the cohort of laboratory staff was expected to be nonparametric i.e., not have a normal distribution, but the D'Agostino-Pearson test for normality was performed to confirm this for each data set. When two categories were analysed, the Mann-Whitney test was used to compare the various points of interest. When more than two categories were analysed for statistical significance, the Kruskal-Wallis test was used.

Ethics

This study was approved by the University of Cape Town Human Research Ethics Committee (HREC reference number: 005/2022). Informed consent was not required for the retrospective use of routine samples as they were de-identified in this study. Only adult participants with capacity to give informed consent were invited to participate in the cohort of laboratory staff.

CHAPTER 3

3. RESULTS

3.1 Anti-nucleocapsid immunoassay

3.1.1 Specificity

A total of 316 de-identified, residual pre-pandemic serum samples (collection date prior to December 2019) were tested to assess specificity of the anti-nucleocapsid immunoassay. Forty historical samples with previous equivocal serological results were included in the 316 samples, to control for false reactivity. All results were valid and of the 316 samples tested, 315 samples were negative. The one sample that did test positive, had a signal/cut-off value (S/CO) of 2.76 i.e. it was a low positive result. The sample was repeated and a similar low positive result was obtained. Interestingly, this singular low positive sample was not one of the 40 samples with historically equivocal serology results. According to the information captured on the NHLS TrakCare database, this sample had a collection date from September 2019, three months before the first reported case of SARS-CoV-2 on 31st December 2019 in Wuhan, China. The sample belonged to a 20-year-old female attending a local clinic who was being investigated for severe anaemia and a bicytopenia (thrombocytopenia and low red cell count). No further clinical history was available for this sample. The calculated specificity of the anti-nucleocapsid immunoassay was 99.7% (95% CI, 98-100).

3.1.2 Sensitivity

A total of 404 de-identified, residual serum or plasma samples with an associated positive SARS-CoV-2 PCR at least 10 days prior was tested to evaluate sensitivity. All results were valid and of the 404 samples, 369 tested positive. The characteristics of the 35 samples that tested negative were analysed. Five of the 35 negative samples were from patients with a positive SARS-CoV-2 PCR less than 2 weeks prior to the collection date of the serum or plasma sample. Of the 30 negative samples that were more than 2 weeks post-PCR positivity, one of them was from a patient with a haematological malignancy and another 16 of them had doubtful SARS-CoV-2 PCR results (Seegene and Cepheid GeneXpert) i.e. triple, double or single PCR gene targets with high cycle threshold (Ct) values (>30), indicating very low levels of viral nucleic acid. The calculated sensitivity of the Roche anti-nucleocapsid immunoassay was 91.3% (95% CI, 88-94). The sensitivity at different time intervals in weeks since SARS-CoV-2

PCR positivity was also examined and a general trend of increased sensitivity after 2 weeks post-PCR positivity was observed (Table 1).

Table 1: Sensitivity of Roche anti-nucleocapsid immunoassay at different time intervals in weeks since SARS-CoV-2 PCR positivity

Weeks since positive PCR	Total no. tested	Positives	Negatives	Percentage Positive (%)
<2	44	39	5	**88.6**
2-3	89	80	9	**89.9**
4-5	74	66	8	**89.2**
6-7	56	51	5	**91.1**
8-9	35	33	2	**94.3**
10-11	46	43	3	**93.5**
≥12	60	57	3	**95**
Overall	**404**	**369**	**35**	**91.3**

Abbreviations: SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2, PCR = polymerase chain reaction

3.1.3 Precision

In accordance with the CLSI EP12 A2, to assess precision, both inter-run and intra-assay, of the qualitative anti-nucleocapsid immunoassay, five replicates of five samples (A-E) with varying values around the assay cut-off of 1 was tested daily for five consecutive days (Table 2). Sample A and B were negative and sample C-E were low positives. Two of the samples were known negatives from the specificity studies, with high S/CO values, close to the assay cut-off of 1. The other three samples were known positives from the sensitivity studies, with low S/CO values, just above the assay cut-off of 1.

Table 2: Raw data (S/CO) of precision studies for the Roche anti-nucleocapsid immunoassay

	DAY 1				
Sample	**1**	**2**	**3**	**4**	**5**
A	0,66	0,65	0,64	0,65	0,65
B	0,72	0,74	0,78	0,75	0,76
C	1,48	1,47	1,49	1,46	1,45
D	1,77	1,83	1,83	1,82	1,78
E	1,91	1,89	1,88	1,87	1,89
	DAY 2				
Sample	**1**	**2**	**3**	**4**	**5**
A	0,64	0,66	0,65	0,64	0,65
B	0,75	0,74	0,78	0,75	0,73
C	1,47	1,47	1,46	1,43	1,48
D	1,8	1,84	1,8	1,76	1,81
E	1,92	1,62	1,93	1,86	1,89
	DAY 3				
Sample	**1**	**2**	**3**	**4**	**5**
A	0,69	0,69	0,66	0,68	0,73
B	0,77	0,79	0,84	0,77	0,76
C	1,52	1,75	1,51	1,55	1,49
D	1,93	1,84	1,96	1,86	1,82
E	2,29	1,62	1,97	1,96	1,8
	DAY 4				
Sample	**1**	**2**	**3**	**4**	**5**
A	0,72	0,81	0,71	0,80	0,87
B	0,85	0,86	0,91	0,87	0,87
C	1,62	1,86	1,64	1,7	1,88
D	2,74	2,01	2,34	2,01	2,22
E	2,36	2,02	2,12	2,08	2,52
	DAY 5				
Sample	**1**	**2**	**3**	**4**	**5**
A	0,70	0,80	0,70	0,83	0,80
B	0,83	0,86	0,93	0,86	0,84
C	1,61	1,83	1,73	1,65	2,00
D	2,76	2,09	2,42	2,02	2,33
E	2,41	2,82	2,17	2,59	2,60

Abbreviations: S/CO = signal/cut-off

Table 3: Analysed data of inter-run precision for the Roche anti-nucleocapsid immunoassay

	Original S/CO	Mean	SD	%CV
Sample A1-A5	0,65	0,7064	0.068696	9.7
Sample B1-B5	0,81	0,8044	0.058330	7.3
Sample C1-C5	1,52	1,6000	0.158013	9.9
Sample D1-D5	1,82	2,0156	0.286497	14.2
Sample E1-E5	1,93	2,0796	0.307395	14.8

Abbreviations: %CV = co-efficient of variation, S/CO = signal/cut-off, SD = standard deviation

The overall co-efficient of variation percentages (%CV) for inter-run precision < 15% (Table 3). The overall %CV for intra-assay precision was <5%.

For the additional, contracted secondary precision study of inter and intra-assay variability in various positive result values, all nine samples (F-N) when repeated after a seven-day interval remained the same. The set of positives samples run a week apart had an inter-assay %CV <5%. The samples that were run in triplicate to assess intra-run variability all had a %CV of <5% (Table 4).

Table 4: Data of secondary precision studies for Roche anti-nucleocapsid immunoassay

	Week 1		Week 2 Replicate 1		Week 2 Replicate 2		Week 2 Replicate 3		Intra-assay precision		
Sample	**Result**	**S/CO**	**Result**	**S/CO**	**Result**	**S/CO**	**Result**	**S/CO**	**Mean**	**SD**	**%CV**
F	P	9,39	P	9,19	P	9,18	P	9,06	9,14	0,072	0,79
G	P	13,83	P	14,09	P	14,11	P	13,96	14,05	0,081	0,58
H	P	50,39	P	50,71	P	50,69	P	50,84	50,75	0,081	0,16
I	P	56,62	P	57,99	P	57,03	P	57,75	57,59	0,5	0,87
J	P	60,04	P	62,44	P	63,5	P	63,72	63,22	0,684	1,08
K	P	97,54	P	98,76	P	97,88	P	99,37	98,67	0,749	0,76
L	P	106,9	P	110,5	P	110,6	P	110,5	110,5	0,058	0,05
M	P	115,7	P	118	P	118,4	P	118,5	118,3	0,265	0,22
N	P	121	P	122,5	P	122,6	P	123,5	122,9	0,551	0,45

Abbreviations: %CV = co-efficient of variation, S/CO = signal/cut-off, SD = standard deviation

3.2 Anti-spike immunoassay

3.2.1 Specificity

A total of 194 de-identified, residual serum and plasma samples with pre-pandemic collection dates (between June and September 2019) were used to assess specificity of the anti-spike immunoassay. All 194 samples tested negative for SARS-CoV-2 anti-spike antibodies, of which 39 samples had pre-pandemic equivocal serology results. The calculated specificity for the Roche anti-spike immunoassay was 100 % (95% CI, 98-100). The one pre-pandemic sample that tested weakly positive on the anti-nucleocapsid immunoassay specificity study was included in this group of anti-spike specificity samples, and the result was negative.

3.2.2 Sensitivity

A total of 384 de-identified, residual serum or plasma samples, with an associated positive SARS-CoV-2 PCR at least 10 days prior, was tested to evaluate the sensitivity of the Roche anti-spike immunoassay. Of the 384 samples, 360 tested positive for anti-spike antibodies. The calculated sensitivity for this assay was 93.8% (95% CI, 91-96). Of the 24 negative samples, seven were serum samples linked to a SARS-CoV-2 PCR that was positive <2 weeks from serum/plasma sample collection date and of the negatives that were >2 weeks PCR-positivity, 15 samples were linked to PCR results with high Ct values.

The 384 samples comprised at least 100 samples representative of each of the first three SARS-CoV-2 infection waves in South Africa (Figure 1). To represent the first wave (circulating wild-type virus), 123 serum or plasma samples were tested, of which 114 were positive. The sensitivity for samples from the first wave was therefore 92.7% (95% CI, 87-97). For the second wave (circulating dominant beta variant), 161 samples were tested, of which 149 were positive. The sensitivity for samples from the second wave was therefore, 92.6% (95% CI, 87-96). To represent the third wave (circulating dominant delta variant), 100 samples were tested, of which 97 were positive. The sensitivity for samples from the third wave was 97% (95% CI, 91-99).

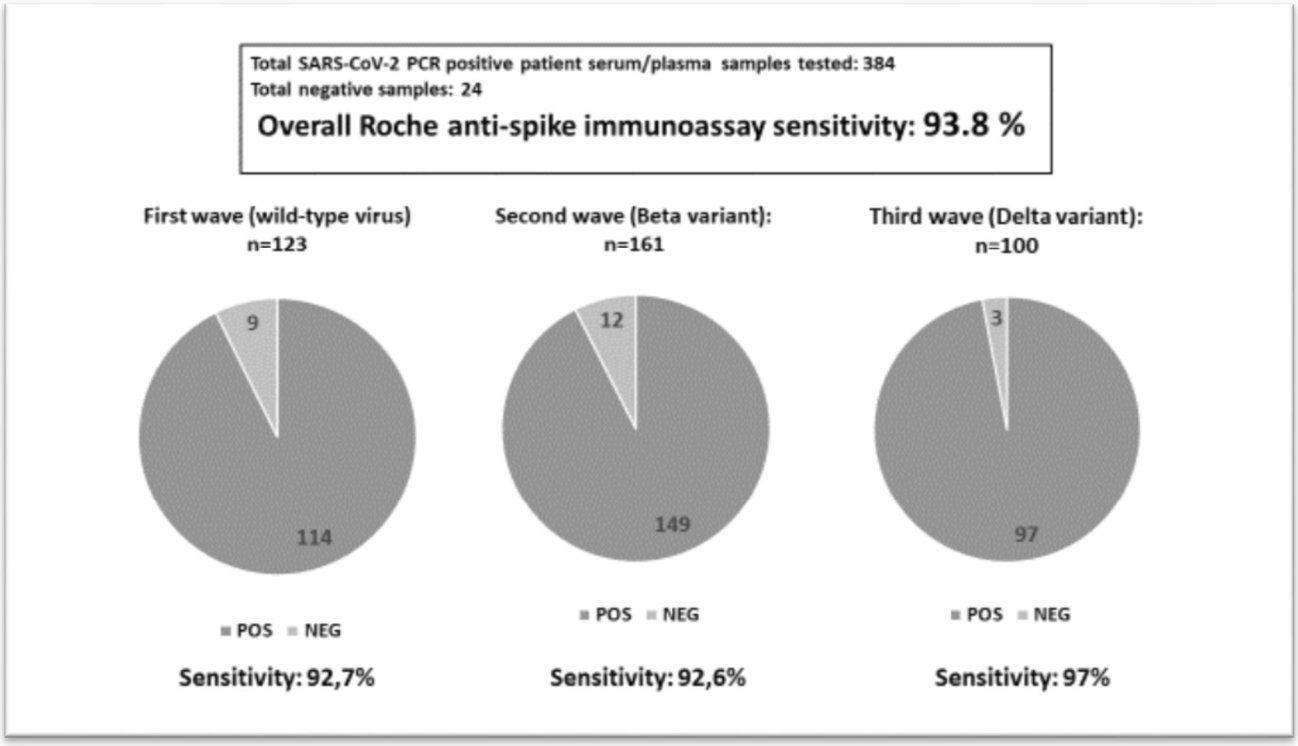

Figure 1: Illustrating sensitivity of the Roche anti-spike immunoassay, both overall and during the first, second and third SARS-CoV-2 infection waves in South Africa. Assay sensitivity remained >90% for all three waves despite mutations in the spike protein in the second and third waves giving rise to circulating dominant variants. Abbreviations: NEG = negative, PCR = polymerase chain reaction, POS = positive, SARS-CoV-2 = severe acute respiratory syndrome coronavirus 2

Sensitivity at different time intervals in weeks since SARS-Cov-2 PCR positivity was also interrogated (Table 5).

Table 5: Sensitivity of Roche anti-spike immunoassay at different time intervals in weeks since SARS-CoV-2 PCR positivity

Weeks since positive PCR	Total no. tested	Positives	Negatives	Percentage Positive (%)
<2	77	70	7	90.9
2-3	116	111	5	95.7
4-5	55	50	5	90.9
6-7	36	34	2	94.4
8-9	13	12	1	92.3
10-11	15	15	0	100
≥12	72	68	4	94.4
Overall	**384**	**360**	**24**	**93.8**

Abbreviations: PCR = polymerase chain reaction

3.2.3 Precision

In accordance with the CLSI EP15 A3 guidelines for precision studies for quantitative assays, five replicates of a single positive and negative kit IQC with assigned target values were run daily over five consecutive days (Table 6). The overall %CV intra-assay precision was <1% and %CV for the inter-assay precision was <5%.

Table 6: Data of precision studies for the Roche anti-spike immunoassay using kit negative and positive controls

Anti-spike Negative Control									
	Target range (U/mL)	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Mean**	**SD**	**%CV**
Replicate 1	0 - 0,4	0,4	0,4	0,4	0,4	0,4	0,4	0,0	0
Replicate 2	0 - 0,4	0,4	0,4	0,4	0,4	0,4	0,4	0,0	0
Replicate 3	0 - 0,4	0,4	0,4	0,4	0,4	0,4	0,4	0,0	0
Replicate 4	0 - 0,4	0,4	0,4	0,4	0,4	0,4	0,4	0,0	0
Replicate 5	0 - 0,4	0,4	0,4	0,4	0,4	0,4	0,4	0,0	0
Anti-spike Positive Control									
	Target range (U/mL)	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Mean**	**SD**	**%CV**
Replicate 1	6,78 - 12,6	7,53	7,77	7,85	7,75	7,78	7,74	0,12116	1,6
Replicate 2	6,78 - 12,6	7,63	7,90	7,87	7,68	7,79	7,77	0,11718	1,5
Replicate 3	6,78 - 12,6	7,69	7,87	7,90	7,59	7,86	7,78	0,13517	1,7
Replicate 4	6,78 - 12,6	7,82	7,77	7,92	7,56	8,05	7,82	0,18229	2,3
Replicate 5	6,78 - 12,6	7,68	7,84	7,87	7,68	7,88	7,79	0,10149	1,3

Abbreviations: %CV = co-efficient of variation, S/CO = signal/cut-off, SD = standard deviation, U/mL = units per millilitre

To strengthen our evaluation of precision for this quantitative assay, a secondary experiment to assess inter-run precision in test values was evaluated using a total of 15 samples. Five samples had a value around the cut-off of 0.8 U/mL (C1-C5), five samples had varying titres of positivity below the first dilution mark of 250 U/mL (P1-P5), and there were five negative samples (N1-N5). These samples were run once daily, for five consecutive days (Table 7). The %CV of antibody titres for all 15 samples was <5% for majority of samples (n=14/15). One sample had a %CV of 17.8%.

Table 7: Data of secondary precision studies for the Roche anti-spike immunoassay showing inter-run variability using samples with known results

Known positive samples	Target value (U/mL)	Day 1	Day 2	Day 3	Day 4	Day 5	Mean	SD	%CV
P1	7,2	6,9	7,5	7,6	7,6	7,6	7,3	0.2767	3.8
P2	17,4	18,0	19,1	19,0	19,5	19,2	18,6	0.5061	2.7
P3	68,4	68,7	72,3	73,7	72,6	75,2	71,9	2.1562	3.0
P4	157,8	162,7	168	167,3	167,6	168	165,4	2.0272	1.2
P5	230,8	230,4	239,7	239,5	242,7	247,8	239,1	5.6679	2.4
Known negative samples	**Target value (U/mL)**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Mean**	**SD**	**%CV**
N1	0,4	0,4	0,4	0,4	0,4	0,4	0,4	0.0000	0.0
N2	0,4	0,4	0,4	0,4	0,4	0,4	0,4	0.0000	0.0
N3	0,4	0,4	0,4	0,4	0,4	0,4	0,4	0.0000	0.0
N4	0,7	0,7	0,7	0,7	0,7	0,7	0,7	0.0125	1.8
N5	0,4	0,4	0,4	0,4	0,4	0,4	0,4	0.0000	0.0
Samples near cut-off	**Target value (U/mL)**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Mean**	**SD**	**%CV**
C1	0,9	0,8	0,9	0,9	0,9	0,9	0,9	0.01862	2.1
C2	1,9	1,8	2,8	2,8	2,8	2,8	2,3	0.40666	17.8
C3	0,9	3,1	3,4	3,5	3,5	3,5	3,3	0.14940	4.5
C4	0,8	0,8	0,8	0,8	0,8	0,8	0,8	0.01301	1.6
C5	1,3	1,2	1,3	1,3	1,3	1,3	1,3	0.04690	3.7

Abbreviations: %CV = co-efficient of variation, S/CO = signal/cut-off, SD = standard deviation, U/mL = units per millilitre

3.2.4 Agreement

During the spike immunoassay verification, we also tested samples from the specificity and sensitivity studies with adequate volume for both anti-spike and anti-nucleocapsid antibodies. The same was not done for wave three samples since vaccination was introduced during this time and we did not have access to patient vaccination history for these samples. Sensitivity, specificity was calculated for each assay and using Stata, agreement and kappa scores were calculated for each of the 3 sets of samples (Table 8). The anti-spike immunoassay performance was superior to the anti-nucleocapsid assay i.e., specificity 100 % vs 99.3%, wave

one, sensitivity 92.6% vs 86.8% and wave two sensitivity 92.5% vs 88.8%. Assay agreement was >90% for all three categories of samples. For wave one samples, there was 90.91% agreement for both assays with a kappa score of 0.51 interpreted as moderate agreement. For wave two samples agreement was 96.3% and a kappa score of 0.78, indicating substantial agreement.

Table 8: Agreement studies

	Specificity/pre-pandemic samples		**Wave one Sensitivity samples**		**Wave two Sensitivity samples**	
	S	N	S	N	S	N
Positive	0	1	112	105	149	143
Negative	146	145	9	16	12	18
Specificity/Sensitivity (%)	100	99.3	92.6	86.8	92.5	88.8
Agreement (%)	99.3%		90.91%		96.3%	
Kappa score	-		0.51		0.78	

Abbreviations: N = nucleocapsid, S = spike

3.2.5 Cohort of laboratory staff

A total of 174 laboratory staff were enrolled in this study. Previous infection with SARS-CoV-2 evidenced by a positive anti-nucleocapsid antibody test was observed in 32% (n=55) of participants, of which 15% (n=8/55) of these anti-nucleocapsid positive participants and 5% overall (n=8/174) indicated no history of natural infection on their questionnaire. With regards to vaccination, 96% (n=167) of participants had received a single dose of a COVID-19 vaccine, of whom a majority of 165 received the Johnson & Johnson adenovirus vector vaccine and two received the Pfizer-BioNTech mRNA vaccine.

Table 9: Demographics of 174 participants in cohort of laboratory staff

	Number of participants
Gender	
Female	123
Male	51
Age	
20-29 years	28
30-39 years	69
40-49 years	35
50-59 years	31
60-69 years	11
History of infection	
Symptomatic	47
Asymptomatic	8
No history of infection	119
Vaccination status	
Vaccinated	167 (Johnson & Johnson vaccine: 165) (Pfizer-BioNTech mRNA vaccine: 2)
Unvaccinated	7

3.2.5.1 Post SARS-CoV-2 vaccine response

Analysis of all the data collected revealed 119 participants who stipulated on their questionnaire that they had no history of natural infection with SARS-CoV-2 and this lack of natural infection was confirmed by a negative anti-nucleocapsid antibody test. Furthermore, of the 119 participants, 115 indicated that they had received a single dose of a COVID-19 vaccine targeting the spike antigen, and four indicated that they had not received a COVID-19 vaccine. All samples were tested for anti-spike antibodies and revealed 99% (n=114) seroconversion amongst the 115 participants who received a single dose of a COVID vaccine. Only one vaccinated participant had no detectable humoral immune response 4 months after

receiving a single dose of the Johnson & Johnson COVID-19 vaccine. The four participants who indicated that they were unvaccinated, tested negative for anti-spike antibodies.

The presence (n=67) or absence (n=48) of post-vaccine symptoms of any severity, did not statistically affect anti-spike antibody titres produced by vaccine recipients (p value = 0.1876). The impact of age and gender on anti-spike antibody titres of vaccine recipients was also examined and found to also have no statistical significance, with a p value of 0.1706 and 0.2654, respectively.

The vaccination of staff began in February 2021. Anti-spike antibody titres post vaccination were analysed in two groups, five months post-vaccination (n=52) versus four months or less post-vaccination (n=63) at the time of this study in July/August 2021. The five-month post-vaccination group had a higher anti-spike antibody titre with a median (IQR) of 105 U/mL (49-345 U/mL) compared to the four-month or less group with median (IQR) of 71 U/mL (29-218 U/mL). However, this was not statistically significant (p value =0.3574).

3.2.5.2 COVID-19 severity and magnitude of immune response

We compared anti-spike antibody titres in the 52 patients who had serological evidence of natural infection against the disease severity that they indicated on their questionnaire (Figure 2). Severity was reported as either asymptomatic (n=7), mild (n=20), moderate (n=20) or severe (n=5). Of the total 174 enrolled laboratory staff, 3% indicated that they had experienced severe COVID-19 for which they required hospitalization. There was no statistical difference in anti-spike antibody titres produced amongst all four categories with a p value = 0.0829 using the Kruskal-Wallis test. However, there was a statistically significant increase in anti-spike antibody titres in participants who experienced symptoms of any severity (n=43) versus asymptomatic participants (n=8) with a p value = 0.0303. All five staff who experienced severe disease, were infected in 2020, prior to the initiation of COVID-19 vaccination at our study site in February 2021. Only 10% (n=17) of the 174 participants had anti-spike antibody titres of ≥ log 4 (Table 9). Of these, all, except one, had a positive anti-nucleocapsid antibody test. Four of the five participants who were hospitalized for severe COVID-19 were included in the 10% mentioned above.

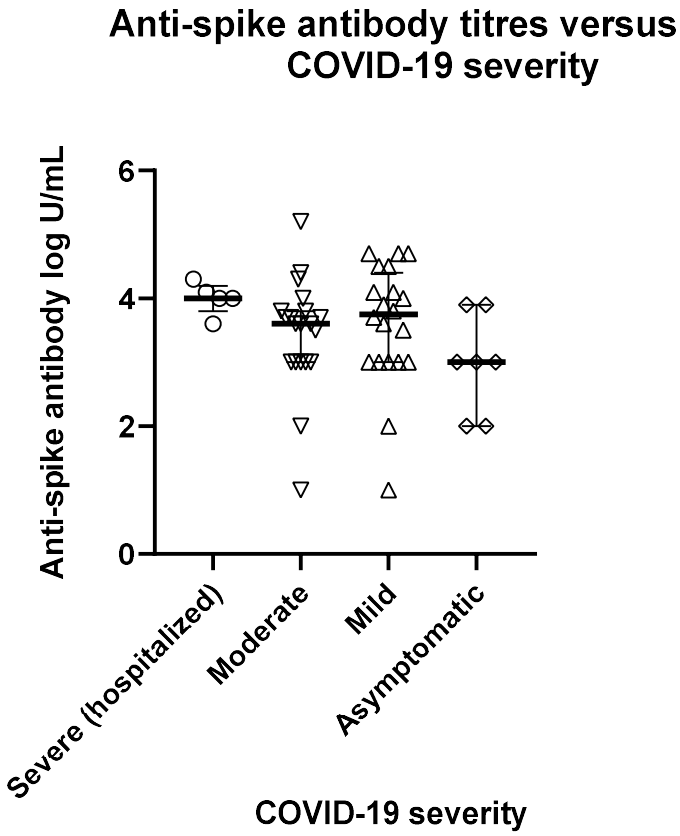

Figure 2: Scatter plot illustrating anti-spike antibody titres in participants with serological evidence of natural infection versus COVID-19 severity. There was a statistically significant increase in anti-spike antibody titres in participants who experienced symptoms of any severity (n=43) versus asymptomatic participants (n=8) with a p value = 0.0303.

Table 10: Characteristics of participants with spike-antibody titres of ≥ log 4

Age	Sex	S titre (U/mL)	S log	N	N S/CO	History of COVID-19	Date of PCR positivity	Disease severity	No. days ill	COVID-19 vaccine	Date vaccinated	Post vaccine symptoms
40	M	156250	5,2	P	78.25	Yes	Jul-21	moderate	9	Y (J&J-sd)	Feb-21	No
59	F	46890	4,7	P	3.18	Yes	Jul-21	mild	2	Y (J&J-sd)	Feb-21	Yes
58	F	45784	4,7	P	87	Yes	Jul-21	mild	2	Y (J&J-sd)	Feb-21	Yes
43	F	44952	4,7	P	22.46	Yes	Jul-21	mild	7	Y (J&J-sd)	Mar-21	No
36	F	35358	4,5	N	0.12	No	n/a	n/a	n/a	Y (J&J-sd)	Feb-21	Yes
41	F	34584	4,5	P	5.44	Yes	Jul-21	mild	10	Y (J&J-sd)	Mar-21	Yes

49	M	28493	4,5	P	59.83	Yes	Jun-21	mild	10	Y (J&J-sd)	Feb-21	Yes
44	F	23730	4,4	P	16.65	Yes	Apr-20	moderate	15	Y (J&J-sd)	Mar-21	Yes
32	M	21695	4,3	P	182.30	Yes	May-20	moderate	14	Y (J&J-sd)	Feb-21	Yes
44	M	21052	4,3	P	215,2	Yes	Dec-20	severe	30	Y (J&J-sd)	Apr-21	Yes
33	M	13242	4,1	P	7,38	Yes	Jul-21	mild	6	Y (J&J-sd)	Mar-21	No
56	F	12011	4,1	P	38.86	Yes	Apr-20	severe	55	Y (J&J-sd)	Feb-21	Yes
28	F	11482	4,1	P	26.30	Yes	Jun-21	mild	3	Y (J&J-sd)	Mar-21	No
61	F	10124	4,0	P	101	Yes	May-20	moderate	21	Y (J&J-sd)	Feb-21	Yes
24	F	9742	4,0	P	3.36	Yes	Jun-21	mild	9	Y (J&J-sd)	Mar-21	No
37	F	9727	4,0	P	39.75	Yes	May-20	severe	3	Y (J&J-sd)	Mar-21	No
33	F	9676	4,0	P	137,5	Yes	Jul-20	severe	15	Y (J&J-sd)	Mar-21	No

Abbreviations: COVID-19= Coronavirus disease 2019, F=female, J&J-sd=Johnson & Johnson vaccine – single dose, M=male, N=anti-nucleocapsid antibody, PCR=polymerase chain reaction, s/co=signal/cut-off, S=anti-spike antibody, U/mL = units per millilitre

3.2.5.3 Anti-spike antibody titres post natural infection and one dose of a COVID-19 vaccine versus one dose of a COVID-19 vaccine only

Of the total 174 volunteering laboratory staff, a group of 45 participants indicated on their questionnaire that they had a history of natural infection with a documented positive SARS-CoV-2 PCR and also indicated that they had received a single dose of a COVID-19 vaccine (hybrid immunity). A further seven individuals were also anti-nucleocapsid antibody positive, indicating prior undocumented SARS-CoV-2 infection. These also received a single dose of a COVID-19 vaccine. This entire group of 52 participants, tested positive for both anti-nucleocapsid and anti-spike antibodies, confirming natural infection. A second group of volunteering laboratory staff, consisting of 115 participants was identified through the questionnaire and dual antibody testing to have no history of natural infection, received one dose of a COVID-19 vaccine, and who were positive for anti-spike antibodies but negative for anti-nucleocapsid antibodies, confirming no previous natural infection with SARS-CoV-2. There was a significant increase in anti-spike antibody titres in the vaccinated and natural infection group (Figure 3) with a median (IQR) of 4565 U/mL (1437-9838 U/mL), compared to the vaccine-only group with a median (IQR) of 83 U/mL (34-239 U/mL). This was a statistically significant finding with a p value < 0.0001. Hybrid immunity was then further separated into participants in whom natural infection preceded vaccination and participants in whom vaccination preceded natural infection (Figure 4). Participants in whom vaccination preceded natural infection had significantly higher anti-spike antibody titres (p value <0.0001).

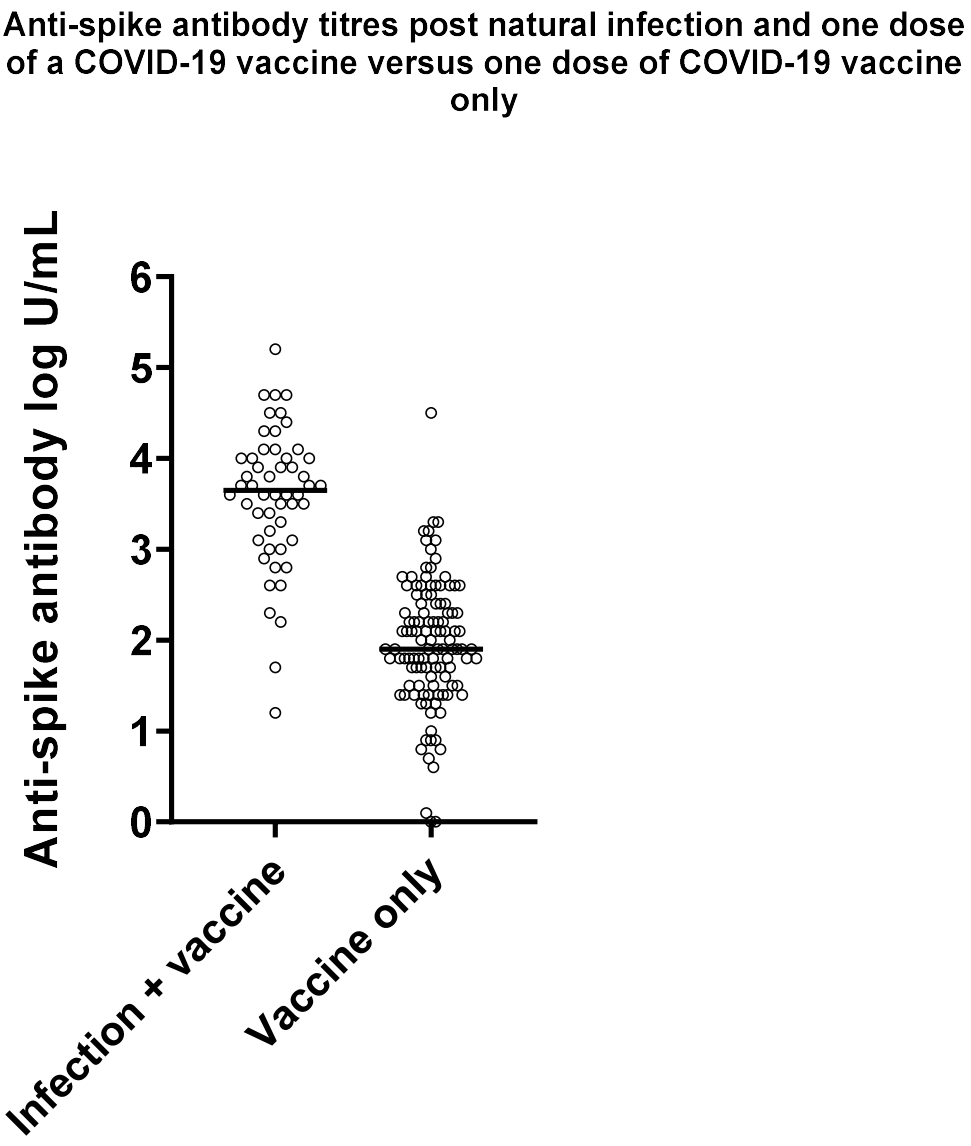

Figure 3: Scatter plot illustrating anti-spike antibody titres post natural infection and one dose of a COVID-19 vaccine versus one dose of a COVID-19 vaccine only. The anti-spike antibody titres were significantly higher in the group that had both natural infection and vaccination.

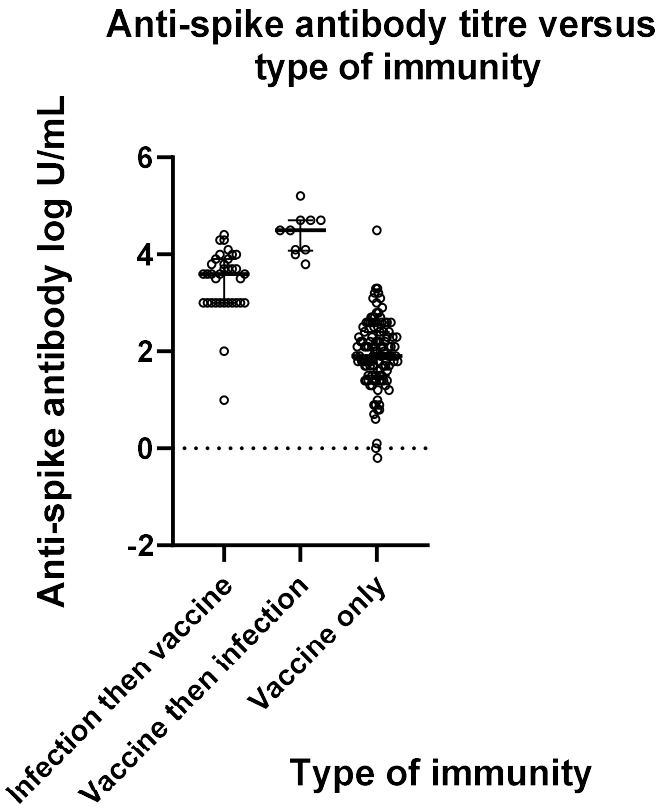

Figure 4: Scatter plot illustrating anti-spike antibody titres versus type of immunity. The anti-spike antibody titres were significantly higher in the group where vaccination preceded natural infection.

3.2.5.4 Effect of time since natural infection on anti-spike and anti-nucleocapsid antibodies

Anti-spike antibody titres were evaluated in the 45 staff members in whom timing of previous SARS-CoV-2 infection was known. Of these 17 staff were infected during the first wave (10-17 months prior to blood collection), 17 in the beta wave (7-9 months prior to sample collection), and 11 were infected during the delta wave (1-2 months prior to samples collection). All staff were anti-spike antibody positive. The anti-spike antibody titres were statistically different amongst all three waves with a p value of <0.0001 using the Kruskal-Wallis test (Figure 4). The anti-spike antibody titre was significantly higher in those who were infected most recently (delta wave) with a median (IQR) of 4.5 log (4-4.7 log) versus the anti-spike antibody titres in both the wild-type and beta wave, median (IQR) of 3.7 log (3.6-4 log) and 3.1 log (2.8-3.7 log), respectively.

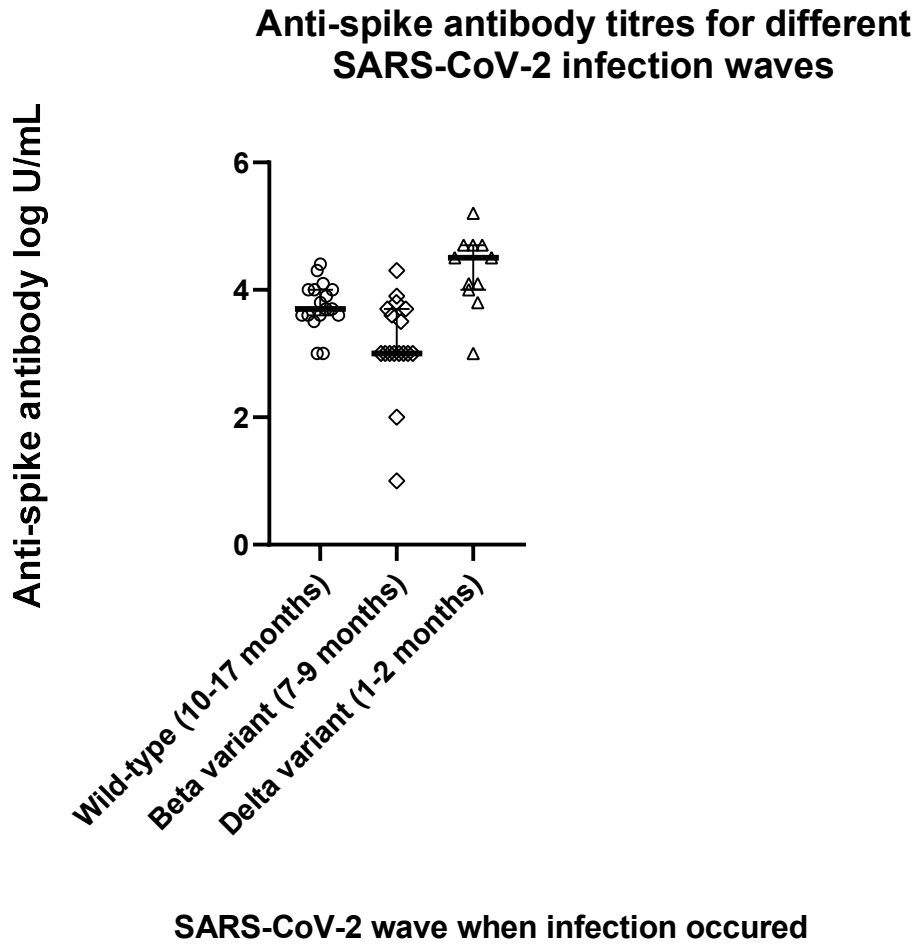

Figure 5: Scatter plot illustrating anti-spike antibody titres post natural infection during different SARS-CoV-2 infection waves in South Africa. The anti-spike antibody titres were significantly higher in those who were infected in the most recent wave (delta wave).

In this group of 45 participants who had a history of COVID-19 PCR positivity 1 to 17 months prior to sample collection, 100% were still positive for anti-nucleocapsid antibody. Furthermore, there was no statistically significant difference in anti-nucleocapsid antibody S/CO values amongst all three waves with a p value of 0.5507 using the Kruskal-Wallis test (Figure 5).

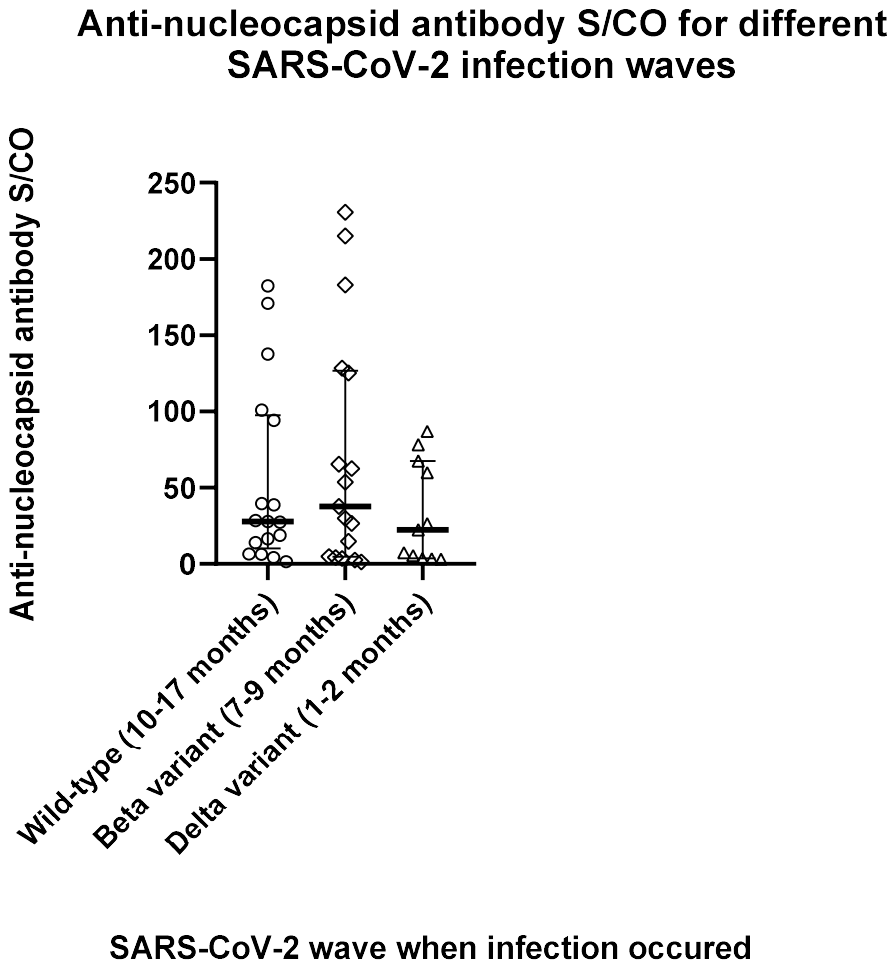

Figure 6: Scatter plot illustrating anti-nucleocapsid antibody S/CO values post natural infection during different SARS-CoV-2 infection waves in South Africa. The anti-nucleocapsid antibody S/CO values were not statistically different amongst all three waves. And 100% of participants with natural SARS-CoV-2 infection 1-17 months ago, were still positive for anti-nucleocapsid antibody at the time of this study.

CHAPTER 4

4. DISCUSSION AND CONCLUSION

4.1. Standard performance data

The acceptance criteria hierarchy used for this study was to meet manufacturer's specifications (40, 41), followed by the WHO target products profile guideline for COVID-19 (37). Performance of both immunoassays in published literature was also examined. After considering that serology only plays an adjunctive role in the diagnosis of SARS-CoV-2, the gold standard being PCR, and that these immunoassays are mainly used for research purposes, our laboratory pragmatically considered the above-mentioned performance targets in constructing our own adapted acceptance criteria for diagnostic use within the NHLS. The adjusted acceptance criteria that our laboratory used for this study was specificity >98%, sensitivity >90% and %CV of precision <20%.

4.1.1 Specificity studies

The specificity of the anti-nucleocapsid immunoassay was 99.7%, which was similar to the manufacturer's claim of 99.8% and higher than the WHO's target of 97%. Specificity of anti-nucleocapsid in published literature was also similar to Favresse et al, Lau et al and the National SARS-CoV-2 Serology Assay Evaluation Group, reporting their specificity as 100%, 99.8% and 98.6% (42-44). The specificity obtained also met our laboratory-adapted acceptance criteria of >98%. The S/CO of the single sample that did test positive, was 2.76 i.e., a low positive and given its collection date of September 2019 (pre-pandemic), this most likely represented false reactivity due to an underlying acute infection or chronic illness such as autoimmune disease or malignancy. During the analytical specificity studies performed by the manufacturer of which 792 potentially cross-reactive serum samples were tested, the 4 that did show cross reactivity were samples representative for an acute Epstein-Barr virus (EBV) infection (n=2), acute cytomegalovirus (CMV) infection (n=1) and SLE (n=1) (40). The nucleocapsid protein in many coronaviruses has been shown to be highly immunogenic and expressed abundantly during infection, therefore cross-reactivity with other coronaviruses was also considered. SARS-CoV-1 and SARS-CoV-2 share the highest nucleocapsid protein sequence homology of 90-91%, however SARS-CoV-1 was last detected during the 2002-2003

outbreak and is not considered a currently circulating respiratory virus (29, 45, 46). Additionally, the manufacturer also included a coronavirus panel consisting of 40 potentially cross-reactive samples from individuals following a PCR-confirmed infection with Coronavirus HKU1, NL63, 229E or OC43, and none were reactive on the anti-nucleocapsid assay (40). This type of experiment was considered but not included in our study because of the lack of paired serum and PCR-positive respiratory samples for other human coronaviruses. However, all 40 pre-pandemic samples, with historical equivocal results included in our specificity study were negative.

The specificity of the anti-spike immunoassay was 100%, which was the same as the manufacturer's claim of 100%, higher than WHO's target of 97% and fulfilled our laboratory-adapted acceptance criteria of >98%. Furthermore, our specificity finding was the same as Lau et al and Jung et al, with 100% and 100%, respectively, and higher than Riester et al with 99.95% (43, 47, 48). The single sample that tested weakly positive in our anti-nucleocapsid immunoassay specificity studies and the 39 pre-pandemic samples with historically equivocal results, were all negative on the anti-spike immunoassay. Thus, considering all the specificity data above, when compared to the anti-nucleocapsid immunoassay, the anti-spike immunoassay's specificity, though similar, was superior overall.

4.1.2 Sensitivity studies

The sensitivity of the anti-nucleocapsid immunoassay was 91.3% (95% CI, 88-94), which was within to the manufacturer's claim between 85.3 to 99.5% (<14 days to > 14 days), slightly lower than the WHO's target of 95% and above our laboratory-adapted acceptance criteria of 90%. Our sensitivity for the anti-nucleocapsid immunoassay was lower than the National SARS-CoV-2 Serology Assay Evaluation Group's sensitivity of 97.2% (44), but similar to Favresse et al with a sensitivity of 92% (42), and within the range of Lau et al who reported 74.4 to 95.1% (<14 days to >14 days) (43).

The sensitivity of the anti-spike immunoassay was 93.8% (95% CI, 91-96), which was within the manufacturer's claim of 87 to 96.6 % (<15 days to > 15 days) but slightly lower than WHO's target of 95%. Riester et al, Jung et al and Lau et al reported a sensitivity of 97.9%, 96% and 79.5 to 95.1% (<14 days to > 14 days), respectively (43, 47, 48). However, the anti-spike immunoassay sensitivity was still above our laboratory-adapted acceptance criteria of >90%.

The analysis of the samples (linked to a positive PCR) that tested negative in the anti-nucleocapsid (n=35) and anti-spike (n=24) immunoassay sensitivity study revealed a combined total of 59 negative samples. Though assay performance may be the cause of these negatives, other reasons such as timing of blood collection, false positive PCRs and biological factors should also be considered.

Twelve of the negative samples were from patients < two weeks since PCR positivity. Time to seroconversion can occur as early as seven days post-symptom onset but the maximum seroconversion has been shown to occur after 14 days (12-14). For the sensitivity study, we used serum samples linked to a positive PCR ≥ 10 days prior to serum/plasma sample collection, therefore samples from patients <14 days post-PCR positivity might not have been positive at the time of blood collection because it was too early following infection. Our sensitivity studies for both immunoassays also demonstrated an increase in sensitivity >2 weeks post-PCR positivity.

During infection waves, the sample PCR positivity rate in our virology lab for waves 1-3 was as high as 30%. Furthermore, the PCR process for SARS-CoV-2 was very hands-on and required manually cutting swabs with repeat-use clippers, repetitively pipetting into 96-well plates and opening and closing of plate covers. These issues, combined with the high volume of samples being processed, many samples with very low Ct values (strongly positive) within runs and staff fatigue, meant that the PCR process was vulnerable to carry-over or contamination events. Of the total 59 negative samples, 31 were linked to PCR results missing gene targets in a multiplex PCR and/or had high Ct values indicating low levels of viral nucleic acid. Though there are other causes of high Ct values such as, PCR inhibition, very early or late infection, poor sample quality and poor sample shipment, contamination has to be considered in cases where high Ct values are obtained as this may be reflective of a false positive PCR. This issue could explain why the 31 samples in the sensitivity studies linked to positive PCRs with high Ct values or missing gene targets tested negative for SARS-CoV-2 antibodies, even though they were supposedly > 14 days post-PCR positivity(49).

Lastly, biological factors that could contribute to non-seroconversion included age, gender, genetics, primary or acquired immune deficiency, malignancies, and immunosuppressive therapy (50, 51). It must also be considered that these high Ct values mentioned above, may be true positives and reflective of low SARS-CoV-2 viral loads in the respiratory tract which

has been linked to a weak systemic immune response resulting in non-seroconversion (52-54).

Two of the samples that tested negative in the anti-nucleocapsid sensitivity studies could have been due to biological causes since both samples were linked to more than one strongly positive SARS-CoV-2 PCR (all gene targets present and low Ct values), one 36 days prior and the other 54 days prior to blood collection. According to the NHLS TrakCare database the former sample belonged to a 30-year-old female and the latter to a 47-year-old male who both had a haematological malignancy, pancytopenia and infection with human immunodeficiency virus (HIV).

While wild-type virus was the dominant circulating virus during South Africa's first wave in early to mid-2020, the Beta variant became the dominant circulating variant during the second wave in late 2020/early 2021, followed by the Delta variant in mid-2021 (55). The Beta variant has three mutations in the RBD of the spike protein (K417N, E484K, and N501Y) which increases the affinity of the viral RBD for its human receptor, ACE-2. The spike protein of the Delta variant has eight mutations, two of which (L452R and T478K) are in the RBD (56). The anti-spike immunoassay uses a recombinant protein representing the RBD of the spike antigen based on the original Wuhan sequence(41). Though much data has been published about the effect of such variants on molecular and neutralization SARS-Cov-2 assays (57, 58), there is a distinct paucity in the literature regarding how these variants affect high throughput antibody immunoassays. Using at least 100 samples to represent each of the first three infection waves in South Africa, our sensitivity studies for the anti-spike immunoassay demonstrated that sensitivity remained >92% in samples from patients infected in all three waves despite mutations in the spike protein of the circulating variants in the second and third wave of infection.

4.1.3 Precision studies

According to the manufacturer's specifications, the overall intra-assay and inter-assay %CV of the precision studies for both anti-nucleocapsid and anti-spike immunoassay were <10% (40, 41). In general laboratory practice, an inter-assay %CV of <15 and an intra-assay %CV of <10 is considered acceptable, others recommend <30 (59-61). For the anti-nucleocapsid immunoassay, the overall %CV for intra-assay precision was <5% and the inter-assay precision

was <15%. For the anti-spike immunoassay, the overall %CV for both intra-assay and inter-assay precision was <5%. The precision studies of both immunoassays met our laboratory-adapted acceptance criteria of <20%. Additionally, for both immunoassays, none of the samples with values nearing the cut-off traversed the cut-off on either side i.e., their qualitative results remained the same as the original throughout the precision study.

There was one sample (C2) in the secondary inter-assay precision study for the anti-spike immunoassay that had an increasing titre during the 5-day study period with an inter-assay %CV for that particular sample of 17.8%. Possible causes include evaporation from samples not being capped timeously, improper sample storage, carry-over events, imprecise pipetting by operator or instrument (60, 61). Because only one sample of all the precision studies was affected, a contamination event or evaporation from prolonged storage whilst uncapped on the instrument, benchtop or fridge is the most likely cause. However, the %CV of this sample was still within our acceptance criteria of <20% and the qualitative value of the sample did not change anytime during the 5-day period.

4.2. Cohort of laboratory staff

A total of 174 laboratory staff were enrolled in this study. Like other healthcare workers around the world, laboratory staff were required to continue working on-site throughout the lockdown periods to keep up with the increase in sample influx across the laboratory services for admitted COVID-19 patients and of course SARS-CoV-2 diagnostic testing. Therefore, aside from transmission events at home, these staff were also susceptible to SARS-CoV-2 transmission and infection from commuting to work, often using public transport, carpooling with other colleagues, and sharing offices, tearooms and work benches.

Based on a positive anti-nucleocapsid antibody test, it was determined that 32% of staff had a history of previous SARS-CoV-2 infection of which, according to our questionnaire, 15% were asymptomatic (5% overall). Wilmes et al showed that asymptomatic index cases play an important role in SARS-CoV-2 transmission with a lower but significant secondary attack rate compared to symptomatic index cases of 0.02 versus 0.04, respectively. Furthermore, on average, asymptomatic individuals were shown to infect 0.6 contacts, only slightly less than the 0.7 infected contacts associated with symptomatic individuals (62). A systematic review and meta-analysis showed that it was difficult to establish exactly what proportion of SARS-

CoV-2 infections were asymptomatic due to the high heterogeneity amongst 130 studies but found the IQR to be 14-50%. The secondary attack rate in contacts of individuals with asymptomatic infection compared with symptomatic infection was reported by this study as 0.32 (95% CI 0.16 to 0.64, prediction interval 0.11 to 0.95, eight studies) (63). Thus, since the national SARS-CoV-2 testing strategy centred on testing symptomatic individuals only and not screening (64), these asymptomatic staff had gone undetected, continued to present to work and possibly served as a source of SARS-CoV-2 transmission within the workplace, albeit less than symptomatic staff.

4.2.1 Post SARS-CoV-2 vaccine response

Amongst our 174 laboratory staff, 96% had received a single dose of a COVID-19 vaccine 2-5 months prior to blood collection for this study, with all but two participants receiving the Johnson & Johnson vaccine. Of the 115 vaccine recipients with no prior serological evidence of past natural infection, 99% were positive for anti-spike antibodies. The one negative anti-spike antibody result belonged to a 52-year-old male who had received a single dose of the Johnson and Johnson vaccine five months prior to blood collection for this study. The participant was known to have diabetes mellitus type 2 for which he was receiving appropriate treatment, however glycaemic control was not known. He also had a history of a malignant tumour on his shoulder in 2018, for which he received radiation and chemotherapy. The lack of anti-spike antibodies in this participant could either be due to him being a primary vaccine non-responder or someone who did initially seroconvert following vaccination but mounted a weak humoral immune response that waned over the 5-month period.

Assuming this staff member was a primary vaccine non-responder, his medical history could have been the contributing factor. It is a well-known fact that diabetes mellitus is associated with impaired innate and adaptive immune responses due to chronic hyperglycaemia and metabolic inflammation. This also relates to why SARS-CoV-2 infection in diabetic patients, particularly those with poor glycaemic control, is linked to an increased risk for morbidity and mortality (65). At present there is no published data regarding seroconversion rates in diabetic patients following vaccination with the Johnson & Johnson vaccine. A study showed that diabetic patients who received a single dose of another adenovirus-based COVID-19 vaccine, ChAdOx1 nCoV-19 (AZD1222), had a significantly lower geometric mean

concentration of antibodies specific for SARS-CoV-2 compared to non-diabetic patients (P<0.001) (66). While Ali et al reported that diabetic patients had lower anti-SARS-CoV-2 IgG (p=0.041) and neutralizing antibody (p=0.036) titres following two doses of the Pfizer-BioNTech vaccine when compared to non-diabetic patients (67). Furthermore, a small study by Pal et al showed that after day 14 of non-severe COVID-19 infection, diabetic patients were more likely than non-diabetic patients to have undetectable SARS-CoV-2 antibodies on a qualitative electrochemiluminescence immunoassay measuring total antibodies ((p=0.019) (68). The long-term effects of radiation therapy and chemotherapy on the immune system, particularly quantitative and functional changes in lymphocyte subsets was likely to also have significantly contributed to vaccine failure in this staff member who had received such therapy just 24 months prior to vaccination(69-73).

Considering the patient was 52 years old, the safety and immunogenicity study of the Johnson & Johnson vaccine for the cohort aged 18-55 years of age was reviewed. At day 29 post-vaccination with a single dose, Sadoff et al reported 99% seroconversion for binding anti-spike antibodies on ELISA and 92% seroconversion for neutralizing antibodies on a viral neutralization assay (VNA) against wild-type SARS-CoV-2. Of note, 82-94% of this cohort produced a robust neutralizing antibody response with titres >100. In terms of cell-mediated immune response, 80% of this cohort demonstrated a Th1-skewed spike-specific CD4+ T cell response as well as a strong CD8+ T cell response. Though spike binding antibodies may not correlate with the cell-mediated immunity, Sadoff et al also showed that the results of the VNA and the spike binding titres on the ELISA in this cohort highly correlated, with a Spearman correlation of 0.86 (p<0.001) (72). Thus, it can be inferred that since this participant was negative for anti-spike binding antibodies, he most likely did not have any neutralizing antibodies either.

It is unfortunate that this staff member had at least two clear risk factors for non-seroconversion following vaccination and at least one known risk factor for poor outcomes following SARS-CoV-2 infection. After being denied a second vaccine despite being provided with evidence by this study showing a lack of vaccine response, this participant sadly demised from COVID-19 in September 2021, likely corroborating a lack of vaccine-induced protective cell-mediated immunity as well. At present, the WHO recommends a second dose of the Johnson and Johnson vaccine for all people with immunocompromising conditions aged 18

years and older and evidence suggests that this dose should be given 1-3 months after the first dose (74). Given our staff member's medical history, whether or not a second dose of vaccine would have made a difference is unknown. A key lesson from this case was that, there is definitely a role for using widely available, high throughput immunoassays like the anti-spike assay evaluated in this study to confirm and monitor vaccine response by looking for surrogate markers like anti-spike binding antibodies in patients with risk factors for non-seroconversion and increased morbidity and mortality. These results can be used to tailor personalized life-saving, vaccine booster schedules for such individuals.

Lastly, the most common adverse effects following the Johnson and Johnson vaccine are local injection site pain, fatigue, headache and myalgia (72). Though our study found no significant effect of post-vaccine symptom presence and severity on the magnitude of vaccine immune response, at present there isn't any published data for comparison. In terms of the effect of gender on magnitude of vaccine response, our finding that this factor was not significant agreed with current published literature (18). Our data did not to show a statistically significant link between age and post-vaccine anti-spike antibody titres. The most likely explanation is that the age of retirement for staff in the NHLS is 65 years, and therefore our data set lacked the representative age group found to have a significant effect in other studies. Age >65 years was identified as a risk factor for lower immune response post-vaccine (75-77).

4.2.2 Hybrid immunity versus vaccination only

It has been recently shown that the strongest SARS-CoV-2 specific immune response elicited in patients, ranked from strongest to lowest begins with hybrid immunity (natural immunity and vaccination), followed by two doses of vaccine, then natural infection, and lastly one dose of vaccine (75, 78-81).

Results from our study corroborated this finding. When we compared 52 vaccinated staff members with serological evidence of past natural infection (hybrid immunity) with 115 vaccinated staff members who had no serological evidence of past natural infection, we found that participants with hybrid immunity had significantly higher anti-spike antibody titres than the vaccine-only group (p value < 0.0001). Furthermore, only 10% of our total study participants had anti-spike antibody titres >4 log. All had hybrid immunity except one outlier

whose spike antibody titre was log 4.5 but who was anti-nucleocapsid antibody negative and had no history of past natural infection. This could be explained by either a false negative anti-nucleocapsid test related to assay sensitivity or a prozone effect, though the latter reason is less likely since individuals with higher anti-spike antibody log values still had detectable anti-nucleocapsid antibodies.

Another study showed infection-naïve patients who received 2 doses of a COVID-19 vaccine had significantly lower total virus-specific neutralizing antibody titres and lower antibody-dependent cellular phagocytosis when compared to patients with hybrid immunity. Amongst the different classes of RBD specific antibodies in the vaccine-only group, anti-RBD IgA was the lowest, likely relating to the lack of mucosal immune stimulation that would normally occur with natural infection. Viral neutralization was also lower against wild-type, alpha, beta and delta variants in the vaccine-only group compared to those with hybrid immunity. Even amongst patients who were >60 years, hybrid immunity still granted a more robust neutralizing antibody response than seen in the vaccine-only group (75).

This above-mentioned study showed there was no significant difference in magnitude of humoral immune response whether natural infection occurred before or after vaccination (75). Our study found that patients who had vaccination first followed by natural infection had significantly higher anti-spike antibodies ($p < 0.0001$) than a previous infection followed by vaccination. The most likely explanation for our finding is that blood collection for our study began nearly six months after vaccine rollout within our laboratory had commenced. Thus, the higher antibody titres observed in this group is likely reflective of a recent immune stimulation from a post-vaccine break-through infection within six months of blood collection.

The greatest real-world value of this finding regarding hybrid immunity, was shown by studies in France and Brazil which reported that hybrid immunity offered the greatest protection from severe COVID-19 following infection with the currently circulating omicron variant. However, protection from symptomatic disease in patients with hybrid immunity against the omicron variant was still low and vaccine boosting only offered a moderate but transient increase in protection from symptomatic disease (82, 83).

Antigen exposure following natural infection significantly increased the quantity, quality, and breadth of humoral immune response regardless of whether it occurred before or after

vaccination (81). And, yet again, anti-spike binding antibody titres correlated with what was being demonstrated by the more in-depth but largely inaccessible and laborious humoral immune studies such as VNA, again making it a reliable and accessible surrogate marker for humoral immune response.

4.2.3 Disease severity and anti-spike antibody immune response

Many asymptomatic infections go unnoticed thus there is little known about the true quantity and quality of their humoral immune response (84). Our study revealed a statistically significant increase in anti-spike antibody titres in participants who experienced symptoms of any severity (n=43) versus asymptomatic participants (n=8) with a p value = 0.0303. Our finding was in accordance with another study that showed asymptomatic individuals had lower antibody levels when compared to those that were symptomatic. However, this study also showed that though lower, the antibodies elicited were still polyfunctional i.e., able to neutralize the virus, activate antibody-dependent cellular cytoxicity (ADCC), and trigger complement deposition albeit less than that of symptomatic individuals (84). Given the above, priming of memory B and T cells likely occurred during asymptomatic infection, boosting this immune response with vaccination could play an important role in this group.

Furthermore, the 17 participants who had the highest anti-spike antibody titres overall (>4 log) had a mixture of COVID-19 severity including mild, moderate, and severe (hospitalized) but four of the five hospitalized cases and none of the asymptomatic cases were amongst these 17 participants.

4.2.4 Effect of time since natural infection on anti-spike and anti-nucleocapsid antibodies

When anti-spike antibody titres post-natural infection were compared to the wave in which the primary infection occurred, we found that the titres were significantly higher in those who were infected in the most recent wave (delta wave) with a median (IQR) of 4.5 log (4.0-4.7 log). This again is likely reflective of a more recent immune stimulating event which was shown in real-world data to be the most important factor in preventing SARS-CoV-2 infection (78). Interestingly, even though the wild-type wave occurred before the beta wave, anti-spike antibody titres were significantly lower in the beta variant wave than titres of the wild-type wave (p=0059) with a median (IQR) of 3.1 log (2.8-3.7 log) and 3.7 log (3.6-4.0 log), respectively. Though there isn't any clear data that shows lower antibody titres in response

to the beta variant in hybrid immunity specifically associated with Johnson & Johnson vaccination, this finding is in accordance with studies that showed neutralizing antibody titres and neutralization breath to be lowest for beta variant when compared to wild-type and delta in vaccine-only and hybrid immunity associated with mRNA vaccines (75, 85, 86).

In the vaccine-only group, spike antibody titres did not decrease five months post-vaccination, in fact the median (IQR) increased from 71 U/mL (29-218 U/mL) at less than five months to 105 U/mL (49-345 U/mL) at five months or more post-vaccination. This in keeping with another study's finding that showed humoral immune response persisted for eight months post-vaccination with a Johnson & Johnson vaccine (87).

Though there is a lack of clear published data on anti-nucleocapsid antibody dynamics over time, our study showed that anti-nucleocapsid antibody S/CO values were not statistically different amongst infections that occurred in the three different waves, and anti-nucleocapsid antibodies showed 100% persistence in participants with a history of natural SARS-CoV-2 infection 1-17 months ago.

4.3 Limitations

Majority of serum and plasma samples chosen for the sensitivity studies of both immunoassays were based on day of PCR-positivity rather than symptom onset as this clinical information was not available. Furthermore, a negative result on either immunoassay does not conclusively rule out the possibility of previous SARS-CoV-2 infection or vaccine response as other components of humoral and cellular immunity were not investigated for this study. False positive PCRs especially due to contamination events cannot be ruled out in cases where corresponding antibody results were negative. After this study was performed, guidelines were updated to advise a second dose of vaccine in those who had received a single shot of Johnson & Johnson vaccine and therefore certain findings in this study may not fully represent the anti-SARS-CoV-2 immune climate responding to the currently circulating omicron variant.

4.4 Conclusion

The Roche Elecsys anti-nucleocapsid and anti-spike immunoassay demonstrated overall robust performance that met our acceptance criteria, and we therefore deem both these assays to be suitable for diagnostic use in our virology/chemistry laboratory within the NHLS

in South Africa. Our cohort of laboratory staff showed that hybrid immunity produced higher anti-spike antibody titres compared to vaccine-only participants and this finding was statistically significant and in keeping with recent findings in multiple other studies. Both assays can be used for seroprevalence studies and to identify donors for convalescent plasma therapy. The anti-nucleocapsid immunoassay assay is valuable in confirming past natural infection in patients with previous asymptomatic infection, previous symptomatic infection where no PCR was done or PCR-negative patients who present to hospital with COVID-19 during the second week onwards of illness. Most importantly, the anti-spike immunoassay can be used as a reliable, cheap and easily accessible surrogate marker of post-vaccine humoral immune response and we recommend using this to confirm and monitor humoral immune response in patients with risk factors for non-seroconversion following vaccination and increased risk for morbidity and mortality following infection with SARS-CoV-2.

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6. APPENDIX 1: Consent Form

Informed Consent Form

Title of Research Project: Evaluation of the performance of two SARS-COV-2 immunoassays

You are invited to take part in a research project studying whether the Roche SARS-CoV-2 anti-spike assay is able to detect vaccine-induced antibody, as well as antibody from natural infection. For this we require you to answer a questionnaire and to donate 5 ml of blood (only 1 tube). Your sample will then be tested with two tests: Anti-spike antibody (positive after vaccine or natural infection) and anti-nucleocapsid antibody (positive after natural infection only). You will be informed of the result if you selected "'yes'' to question 11 on the questionnaire. Participation is entirely voluntary and you may choose not to participate in this study. You can withdraw your permission for the use and sharing of your health information at any time.

Please contact Dr Michelle Naidoo (081 4322398) or Prof Diana Hardie (079 1947446) if you wish to withdraw from the research study or have any questions. Information collected will be treated as confidential and protected. If your information is used in a publication/medical congress, your identity will remain anonymous.

Certificate of Consent

Declaration by participant

By signing below, I …… agree to take part in a research study entitled:
Evaluation of the performance of two SAR-COV-2 immunoassays

I declare that: I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable; I have had a chance to ask questions and all my questions have been adequately answered; I understand that taking part in this study is by my own choice and I have not been pressurized to take part; I understand that no reimbursement will be provided to me for my participation; I may choose to leave the study at any time and will not be penalized or prejudiced in any way; I understand that any left-over sample will be stored for a maximum of 5 years and may be used for other laboratory-related research that would help NHLS to improve the diagnostic services it provides to South Africa.

Signed at (*place*) ..... on (*date*) …….. 2021

…… ……

Signature of participant Signature of witness

Declaration by investigator

I *(name)* …… declare that: I explained the information in this document to ……..and encouraged him/her to ask questions and took adequate time to answer them. I am satisfied that he/she adequately understands all aspects of the research, as discussed above I did/did not use an interpreter. (*If an interpreter is used then the interpreter must sign the declaration below.*

Signed at (*place*) ..... on (*date*) …….. 2021

…… ……

Signature of investigator Signature of witness

APPENDIX 2: Questionnaire

Participation in a study to verify the performance of the Roche Elecsys anti-spike and anti-nucleocapsid SARS-CoV-2 antibody immunoassays

DEPARTMENT	
NAME	
GENDER	
DATE OF BIRTH	
AGE	
CONTACT NUMBER	

Virology and chemistry are evaluating the performance of two new serology assays on the Roche Cobas instrument: Anti-spike antibody (positive after vaccine or natural infection) and anti-nucleocapsid antibody (positive after natural infection only).

As part of the validation we would like to assess how well the assay is able to detect vaccine induced antibodies versus antibody responses from natural infection. We are therefore looking for vaccinated and unvaccinated volunteers to donate a single blood sample which will be used in the evaluation. Participation is entirely voluntary. The results will be made available to those who would like to know. However, at this stage it is unknown whether results obtained from this assay are a useful indication of vaccine response. Thus these results are for interest only and **should not** be used for management purposes or to abandon COVID safety regulations. You will be asked to sign the attached consent form.

Any questions you may have can be directed to Dr Michelle Naidoo (081 4322398 or Reg Room 2 or michelle.naidoo@nhls.ac.za) or Prof Diana Hardie (079 1947446 or diana.hardie@nhls.ac.za) in virology. In order for us to gain insight into how well the assays work to identify natural infection or vaccine induced antibody, we would be grateful if participants could provide us with answers to the following questions:

1. Have you had COVID? **Y** **N**

2. If yes, specify the date (s) of your PCR test:

3. Was your infection: **mild** **moderate** **severe** **asymptomatic**

(hospitalization)

Number of symptomatic days: _____

4. If asymptomatic, how was your infection status determined?

   Positive PCR / Positive antibody test

5. Have you had any confirmed SARS-COV-2 positive contacts for which you were required to be quarantined? **Y** **N**

   If yes, specify the date (s) of your encounter(s):

6. Have you had a COVID antibody test before?

7. Have you been vaccinated?

8. Which vaccine did you get?

9. Date of vaccine administration:

10. Did you experience any symptoms post vaccination? **Y** **N**

    If yes, grade severity: **1+** **2+** **3+**

11. Would you like to be informed of the antibody test result? **Y** **N**