DEVELOPMENT OF AN IN-HOUSE SEROLOGICAL TEST TO DETECT SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS-2 (SARS CoV-2) USING RECOMBINANT NUCLEOCAPSID PROTEIN

BY

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DECLARATION

I Muuba Chiyumbabeenzu do declare that this dissertation represents my own work. This work has not been done anywhere else in Zambia, neither has it been submitted for any qualification at the University of Zambia or any other university.

Signature…… Date……

CERTIFICATE OF APPROVAL

This dissertation by Muuba Chiyumbabeenzu has been approved as fulfilling the requirements for the award of the degree Master of Science in One Health Laboratory Diagnostic Sciences by the University of Zambia.

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ABSTRACT

Coronavirus disease-19 (COVID-19) is a major global concern for public health. This laboratory-based study was conducted at the University of Zambia, School of Veterinary Medicine (UNZA-Vet) laboratory. The study aimed at developing an in-house test to detect SARS-CoV-2 using the recombinant SARS-CoV-2 nucleocapsid gene. Recombinant based protein immunoassays are frequently used in veterinary medicine to detect antibodies against different viruses. There is no in-house recombinant protein-based immunoassay (IFA/ELISA) for detection of SARS-CoV-2 antibodies in Zambia. The N gene from the full SARS-CoV-gene was obtained by PCR using N gene primers tagged with *SacI* and *SphI* restriction ends. The N gene was cloned into a vector plasmid pCAGGS-MCS. The cloned N gene was transformed into competent DH5α cells. The N gene was purified and sequenced to ensure that there were no mutations within the gene and then transfected into VERO-E6 cells for protein expression. Archived serum samples (ten samples) of individuals previously infected with COVID-19 were tested for COVID-19 using OnSite® Rapid Test. The recombinant NP expressing cells were used as an antigen for an in-house immunofluorescent antibody test (IFA). The serum samples that tested positive on the rapid test were subjected to IFA using the recombinant N antigen prepared. None of the positive testing sera showed positive results on the assay. This could have been due to the expression system used which expressed a non-reactive protein. Another possible reason could have been the serum samples having low reactivity to the recombinant protein because the time frame between infection and when the samples were collected was not known. Hence, validation of this assay could not be conducted. The techniques used in recombinant antigen development, for detection of antibodies can potentially be applied in manufacturing of serological diagnostic kits. The findings of this

study indicate that there is need for further investigations into the development of serological tests for sero-surveillance of COVID-19.

DEDICATION

I dedicate this thesis to my amazing parents; Mrs. Reginah Muyanda Chiyumbabeenzu and Mr. Thom Chiyumbabeenzu. Your support and love have been the driving force propelling me through the peaks and valleys of my educational journey. I am totally grateful for the support and motivation rendered to me.

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LIST OF SYMBOLS AND ABBREVIATIONS

COVID	Corona Virus Disease
DTH	Diaminobenzidine Tetra Hydrochlorides
ELISA	Enzyme Linked Immunosorbent Assay
HCOV	Human Coronavirus Disease
IFA	Immunofluorescence Assay
IgM	Immunoglobulin M
IgG	Immunoglobulin G
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SARS CoV-2	Severe Acute Respiratory Syndrome Coronavirus Two
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Coronavirus disease 19 (COVID-19), is a novel infectious disease that emerged from Asia in 2019.

The causative agent is a novel corona virus officially named Severe Acute Respiratory Coronavirus-2 (SARS-CoV-2). Due to a rapid rise in the number of cases, WHO declared COVID-19 a world pandemic on 11th March, 2020 (Nguyen, Skelly and Goonawardane, 2021).

The COVID-19 pandemic has significantly impacted various aspects of human health, including physical and psychological wellbeing. The pandemic has impacted mental health leading to anxiety and depression (Afrashtehfar *et al.*, 2023) . Additionally, as a result of COVID-19, a number of adverse changes have occurred in the global economy. Due to the coronavirus pandemic, planes were grounded, scientific, cultural and sports events were canceled and factories were closed. Coronavirus has therefore impacted the entire global economy (Afrashtehfar *et al.*, 2023).

Central to preventive and other treatment options available is to correctly identify individuals exposed to the virus. SARS-CoV-2 offers another challenge as a bigger proportion of individuals infected may not develop symptoms or only become mildly symptomatic especially dependent upon the type and variant circulating at that particular time. Consequently, serological tests come in handy to accurately assess individuals' exposure status in the community. The significance of determining the extent of exposure to COVID-19 is that it can help us assess the immune status of the population. While SARS-CoV-2 serologic testing is not intended to diagnose or rule-out infection, the results from sero-surveys are key to understanding the extent of exposure to SARS-

CoV-2 in various populations including health workers, first responders and the elderly. This is essential for understanding transmission dynamics, assessing the effectiveness of control measures put in place by policy makers and guiding future intervention strategies (Mullins *et al.*, 2020).

There are limited reports on sero -surveillance of SARS-CoV-2 in Zambia; therefore, the true extent of exposure is unknown. As such comprehensive surveillance would clarify the extent of virus distribution. Surveillance forms the basis for response in disease outbreaks and assays to detect virus-specific antibodies are important to understand the prevalence of infection and the course of the immune response. To carry out such an exercise, a simple, rapid, and safe diagnostic and surveillance tool is required (Kajihara, Dautu and Saasa, 2018).

Currently the diagnosis of COVID-19 is based on tests that confirm the presence of SARS-CoV-2. The gold standard for determination of SARS-CoV-2 infection is real-time reverse transcription polymerase chain reaction (PCR). However, despite the high sensitivity and specificity of the RT-PCR, the test cannot be used for post-acute phase detection of the virus and its costs are high (Gómez, 2020). Rapid antigen and antibody detection kits are used for COVID-19 diagnosis. However, they are not particularly accurate in detecting asymptomatic cases (Dhamad and Abdal Rhida, 2020). Commercially available lamina flow assays targeting the N and S protein are currently being utilized for sero-surveillance studies (Nkuba *et al.*, 2022).

Population based sero-surveillance is important to complete data on the reported cases of SARS-CoV-2 infection to assess the real extent of the epidemic and to enable decision makers to adjust public health response measure. Several serological surveys of COVID-19 have been carried out in Africa and have shown variable results of COVID-19 prevalence with majority of cases being asymptomatic (Nkuba *et al.*, 2022).

tSARS-CoV-2 nucleocapsid protein is a core protein of the virus .It binds to the virus genomic RNA to form ribonucleoprotein complex. In addition to assembly, the N protein has various functions including roles in viral mRNA transcription and replication, cytoskeletal tissue and immune regulation. In addition, N protein can induce humoral and cellular immune response which gives it potential to be used for diagnostic purposes (Wu *et al.*, 2023). This renders the N protein a useful protein in the diagnosis of SARS-CoV-2.

Studies have shown that the N protein is highly conserved in different variants of SARS-CoV-2 which makes it suitable for detecting evolving variants of SARS-CoV-2. This implies that the assay can be used for detecting different variants of SARS-CoV-2.

IFA utilizes infected cells immobilized on a microscope slide which acts as an antigen and bound antibodies of a sample (serum) are detected by a fluorophore-labelled secondary (anti immunoglobulin) antibody. The antigen antibody binding is visualized under a fluorescence microscope in a dark environment (Hedman and Nurmi, 2021).

The aim of this study was the development and validation of an in-house SARS-CoV-2 nucleocapsid protein for sero-surveillance of COVID-19 using Immunofluorescence assay (IFA). Such an assay can potentially be applied in sero-surveillance studies to assess the proportion of individuals who have been exposed to the virus and also for assessment of the immune status of a population.

1.2 Statement of the Problem

COVID-19 has spread to all parts of the Zambia. However, information on the true extent of the disease in terms of people who have been exposed is scantly known. Moreover, some individuals with COVID-19 present as asymptomatic individuals and after the virus has cleared, it is not

possible to detect the virus using PCR and so serological tests such as immunofluorescence assay (IFA) are needed.

Prices and supply of antibody tests is unpredictable and can go as high as 170$ which is about K4,250 for 25 tests (CTK, biotech). To overcome this challenge, there is need to develop tests that are relatively cheaper that will detect antibodies to SARS-CoV-2. There is no indigenous/locally produced system that can be used for post exposure or post-acute phase detection of antibodies to the virus. A local system for detection of SARS-CoV-2 exposure, is crucial because it would be an affordable and easy to prepare system for surveillance and post exposure investigations. This can result in reducing expenses which come with purchasing expensive kits for surveillance studies.

1.3 Study Justification

Immunofluorescence assay is less costly, and reagents are easier to access. Additionally, IFA is a simple and fast surveillance tool which can be used for surveillance of COVID-19 even after the epidemic has ended or the prevalence of infection is low. IFA slides have an advantage in that they can be stored for a long period of time and can be transported to remote areas.

The use of a recombinant antigen (Nucleocapsid protein -N protein) has an advantage in that the N protein is immunogenic and elicits an immune response quickly and it is also conserved, therefore; the system has the potential to detect the different variants of SARS-CoV-2. The N protein of SARS-CoV-2 is also easy to clone in prokaryotic and eukaryotic expression plasmids as shown in other studies and this renders the tool convenient for serological assays.(Djukic *et al.*, 2021).

1.5 Study Objectives

1.5.1 General Objective

The general objective of this study was to develop and validate an in-house SARS-CoV-2 serological test that uses recombinant nucleocapsid protein for sero-surveillance of COVID-19 in Zambia**.**

1.5.2 Specific Objectives

i. To clone SARS-CoV-2 N gene (in-vitro) in *E. coli* and express SARS-CoV-2 recombinant N protein in mammalian cells (Vero- E6 cells).

ii. To develop and validate the in-house IFA system for detection of SARS-CoV-2 antigen in serum using recombinant N protein.

1.5.3 Hypothesis

Is it possible to develop an in-house SARS-CoV-2 Serological test that uses recombinant nucleocapsid protein for sero-surveillance of COVID-19 in Zambia?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Human Coronaviruses

SARS-CoV-2 is a novel, zoonotic positive sense single stranded RNA beta coronavirus. The sub family includes SARS-CoV-2 and MERS- CoV and other SARS like viruses of bats (Chin *et al.*, 2020) . The highly transmissible pathogenic virus emerged in Wuhan China in late 2019 causing a pandemic of acute respiratory disease named coronavirus disease 2019, which spread fast all over the world posing an extraordinary threat to global public health (Rauf *et al.*, 2020) . The pathogen was later spotted to be a novel beta corona virus and was provisionally named 2019 novel coronavirus (2019-n CoV) (Wu, Chen and Chan, 2020).

Coronaviruses are not new to human beings and most of them produce mild respiratory diseases in human. They can infect a broad range of vertebrates, including bats, birds, pangolins, snakes, mice, and humans. Recently, the virus has developed the ability to initiate an epidemic by adapting to humans via zoonotic transmission (Sharma, Farouk and Lal, 2021) .

There are six CoVs known, out of which severe acute respiratory syndrome CoV (SARS-CoV) and Middle East respiratory syndrome CoV (MERS-CoV) caused outbreaks in 2002 and 2012, respectively. In 2002, SARS-CoV emerged in China and infected 8422 persons leading to the death of 916 individuals. Later, MERS-CoV appeared in the Arabian countries and infected around 1800 humans (Zhu *et al.*, 2020). The seventh CoV caused large epidemic affecting almost all countries across the globe. As compared with SARS-CoV and MERS-CoV, SARS-CoV-2 is spreading faster and the number of deaths is multifold higher (Kadam *et al.*, 2021).

The sequencing of the 2019 novel virus revealed that the novel virus had most proteins homologous to SARS-CoV. Thus, it was officially designated as SARS-CoV-2 by the International Committee on Taxonomy of Viruses (ICTV) and since then, suggestion for a distinct name was proposed as human coronavirus 2019 (HCoV-19). Similar to SARS coronavirus (SARS-CoV-1), HCoV-19 can cause severe respiratory illness and significant mortality among those over 60 years old with chronic conditions (de Haan, Vennema and Rottier, 2000).

The emergence of SARS-CoV and MERS-CoV and SARS-CoV-2 shows that infectious diseases caused by coronaviruses are serious. Due to frequent human-animal contact, it is probable that mutated novel corona viruses will continue to appear with harmful consequences to human health (Rauf *et al.*, 2020).

2.2 Structure of SARS-CoV-2

Coronaviruses are spherical, enveloped, positive sense unsegmented viruses with club shaped spikes projecting from the surface (Wang *et al.*, 2020). The severe acute coronavirus -2, belongs to order *Nidovirales*, family *Coronaviridae*, subfamily *Orthocoronavirinae*, genus *Betacoronavirinae* and subgenus *Sarbecovirus*. Genetic recombination within members of same or different genera leads to increased genetic diversity, causing spill over to other species and result in the emergence of novel viruses (Nguyen *et al*., 2021).

SARS-CoV-2 3" terminal contains four major structural proteins which are the spike protein , envelope protein , membrane protein and nucleocapsid protein (N) all required for the structurally complete viral particle (Ahmadpour and Ahmadpoor, 2020).

The nucleocapsid protein (N) forms the capsid outside the genome and the genome is further packed by an envelope which is associated with the membrane protein (M), spike protein (S), and

envelope protein (E) (Michel *et al.*, 2020). The SARS-CoV-2 nucleocapsid protein is one of the most predominantly expressed protein by the virus. It is synthesized in the cytoplasm and the N protein ranges from 43 to 50 KDa. Amino acid sequence of SARS-CoV-2 N protein is approximately 90% identical with SARS-CoV N protein (Kadam *et al.*, 2021).

The N protein has two main domains (N-terminal and C-terminal) that can bind to SARS-CoV-2 RNA forming the long, flexible, helical viral nucleocapsid (Chang *et al.*, 2006) and interacts with the M protein during viral assembly (Troyano-Hernáez, Reinosa and Holguín, 2021). It is considered to be a multifunctional protein since it is required for optimal SARS-CoV-2 replication, it enhances the efficiency of virus transcription and assembly, and it plays an important role in viral pathogenesis, triggering the host response to viral infection (McBride, van Zyl and Fielding, 2014). The viral nucleocapsid (RNA + N protein) is synthesized in the cytoplasm, whereas the other structural proteins, i.e., S, M, and E proteins, are transcribed and translated in the endoplasmic reticulum and transported to the Golgi apparatus (Ahmadpour and Ahmadpoor, 2020). Multiple studies have shown that antibodies created against N protein of SARS-CoV-2 are highly immunogenic and abundantly expressed during infection (Kadam *et al.*, 2021).

The spike protein is located on the surface of the viral particle and it mediates attachment to the host receptor. It allows viral entry by attachment. The membrane protein is the most abundant structural protein in the virion and plays a major role in assembly, participating in E assembly and N attachment to the viral genome (de Haan, Vennema and Rottier, 2000) .

The E protein is the less abundant protein in the virion, but essential for correct virus assembly and particle release through interaction with the M protein (Zhu *et al.*, 2020). This protein is involved in critical aspects of the viral life cycle and CoVs lacking the E protein make promising vaccine candidates. The envelope protein facilitates assembly and release of the virus, and the

ion channel activity in SARS-CoV-2 envelope protein is required for pathogenesis (Zhang and Cahalan, 2007).

2.3 Transmission of SARS-CoV-2

SARS-CoV-2 appears to be less lethal than SARS-CoV and MARS-CoV, but its transmissibility is higher (Cevik *et al.*, 2020). The primary route of transmission is through respiratory droplets, but aerosol, direct contact with contaminated surfaces, and fecal–oral transmission was also reported during the SARS epidemic (Cevik *et al.*, 2020). Direct transmission by respiratory droplets is reinforced by productive SARS-CoV-2 replication in both the upper respiratory tract and the lower respiratory tract, and the increasing number of reports indicating human-to-human spread among close contacts exhibiting active coughing (Harrison, Lin and Wang, 2020).

The risk of infection substantially increases in enclosed environments compared with outdoor settings (Cevik *et al.*, 2020). The virus remains viable for many days on smooth surfaces and at lower temperature and humidity, thus, transferring infection from contaminated surfaces to the mucosa of eyes, nose, and mouth via unwashed hands is a possible route of transmission. This route of transmission may contribute especially in facilities with communal areas, with increased likelihood of environmental contamination (Chin *et al.*, 2020) . However, SARS-CoV-2 are readily inactivated by commonly used disinfectants, emphasizing the potential value of surface cleaning and handwashing (Cevik *et al.*, 2020).

2.4 Pathogenesis and clinical signs of COVID-19

Once SARS-CoVs enters the host via the respiratory tract, airway and alveolar epithelial cells, vascular endothelial cells and alveolar macrophages are among their first targets of viral entry (Harrison, Lin and Wang, 2020). The first step in infection is virus binding to a host cell through

its target receptor. The S1 sub-unit of the S protein contains the receptor binding domain that binds to the peptidase domain of the angiotensin-converting enzyme 2 (ACE 2) which is the host target receptor (Arons *et al.,* 2020). Active replication and release of the virus in the lung cells leads to non-specific symptoms such as fever, myalgia, headache, and respiratory symptoms (Cevik *et al.*, 2020).

Furthermore, there is now evidence for non-symptomatic/pre-symptomatic spread of SARS-CoV-2. This finding underscores the ability of SARS-CoV-2 to colonize and replicate in the throat during early infection . Understanding the relative importance of cryptic transmission to the current COVID-19 pandemic is essential for public health authorities to make the most comprehensive and effective disease control measures, which include mask wearing, contact tracing, and physical isolation (Harrison, Lin and Wang, 2020).

Patients with COVID-19 can experience a wide range of clinical manifestation from no symptoms to critical illness (Arons *et al.,* 2020). Patients with mild infection may exhibit a variety of signs such as cough, sore throat, malaise, headache (Arons *et al.*, 2020).

COVID-19 might present different degrees of severity from mild or even asymptomatic carriers, even to fatal cases. The most common complications include pneumonia and acute respiratory distress syndrome (Harrison *et al*., 2020) . Fever, dry cough, muscle weakness, and chest pain are the most prevalent and typical symptoms of COVID-19 (Harrison *et al*., 2020). However, patients might also present atypical symptoms that can occur alone, which might indicate the possible SARS-CoV-2 infection (Harrison *et al.,* 2020).

2.5 Diagnosis

Knowledge of available diagnostic methods of SARS-CoV-2 is fundamental for early diagnosis as well as monitor current cases and identify new cases. The reliable, early and accurate diagnosis is very crucial to provide medical help to the infected individual and help policy makers prevent spread of the disease, in the process of protecting life (Prasad and Yadav, 2020b).

Diagnosis of COVID-19 is primarily by detecting SARS-CoV-2-specific RNA by nucleic acid testing (NAT), but this has limitations, including the possibility of false-negative results due to low viral load in patients with minimal disease, inadequate respiratory tract sampling or mutations in the target sequence, and false-positive results due to contamination or nonspecific amplification (Harrison, Lin and Wang, 2020).

2.5.1 Molecular detection

Molecular diagnosis of COVID-19 primarily relies on the detection of RNA for SARS-CoV-2 virus. Detection of viral proteins is also useful although it has not yet been applied to the diagnosis of COVID-19 (Feng *et al.*, 2020).

Molecular diagnostic assays are a major group of tests used to diagnose COVID-19. Currently, the detection of SARS-CoV-2 RNA by reverse transcription polymerase chain reaction (RT-PCR) is the most widely used method. Other diagnostic molecular methods, including CRISPR-based assays, isothermal nucleic acid amplification methods, digital PCR, microarray assays, and next generation sequencing (NGS), are promising alternatives (Feng *et al.*, 2020).

Studies have shown that SARS-CoV-2 RT-PCR assays have a detection limit below 10 copies/reaction (Habibzadeh *et al.*, 2021) . Low viral loads at the very early or very late stages of infection can lead to false-negative results. Mutations in primer target regions in the virus genome

can also lead to false-negative results (Habibzadeh *et al.*, 2021) . Overall, RT-PCR is the most commonly used method to diagnose COVID-19 due to its high sensitivity and specificity and also to its ability to process large numbers of samples. However, its widespread use is hindered by its requirement for expensive laboratory instruments and skilled laboratory personnel (Prasad and Yadav, 2020a).

The three molecular methods for processing viral RNA; Real time Reverse-Transcription Polymerase Chain Reaction (PCR), Isothermal amplification, and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) use the Centers for Disease Control and Prevention (CDC)-recommended protocol for specimen collection from COVID-19 patients (Dhamad and Abdal Rhida, 2020).

2.5.2 Serology (ELISA,IFA)

A study was conducted to develop an indirect in-house immunofluorescent assay in order to assess the serological status of COVID-19 patients in Marseille, France. In this study, performance of the IFA was compared to a commercial ELISA IgG kit. 888 RT-qPCR-confirmed COVID-19 patients' serums and 350 controls including 200 sera collected before the pandemic, were tested. In this study, a clinical SARS-CoV-2 isolate was used as the antigen. The researchers found that the specificity of the assay was 100% for IgA, 98.6% for IgM and 96.3% for IgG, after testing a series of negative controls. The in-house IFA presented a substantial agreement (86%) with ELISA EUROIMMUN SARS-CoV-2 IgG kit (Cohen's Kappa = 0.61) (Edouard *et al.*, 2021).

A study by Michel et al reported on the development and evaluation of an in-house immunofluorescence assay IFA for SARS-CoV-2 to detect anti-SARS-CoV-2 antibodies. An in-house immunofluorescence assay (IFA), two ELISA kits (EUROIMMUN R ELISA SARS-CoV-2 IgG and NovaLisa R SARS-CoV-2 IgG and IgM and two lateral flow assays (T-Tek R SARS-

CoV-2 IgG/IgM -antibody Test Kit and Sure Bio-tech R SARS-CoV-2 IgM/IgG Antibody Rapid Test) were compared on 40 serums from RT-PCR-confirmed SARS-CoV-2 infected patients and 10 SARS-CoV-2 RT-PCR negative subjects as controls. The researchers found that control subjects tested negative for SARS-CoV-2 antibodies with all five systems. Estimated sensitivities varied from 35.5 to 71.0% for IgG detection and from 19.4 to 64.5% for IgM detection. For IgG, in-house IFA, EuroImmun, T-Tek and NovaLisa displayed 50–72.5% agreement with other systems (Michel *et al.*, 2020).

Maryam Ranjbar reported on the development of a recombinant N protein for the detection of IgG and IgM antibodies to SARS-CoV-2. The recombinant N protein was expressed, purified, and used in an ELISA system. Thereafter, sera samples for COVID-19 patients, as well as sera samples from healthy volunteers, along with sera samples from non-COVID-19 patients were examined by the ELISA (Ranjbar *et al.*, 2022). The researchers found that ELISA sensitivity and specificity were system was between 91.04 and 92.53% for the detection of IgG and IgM antibodies and 98.21 and 97.32%, respectively (Ranjbar *et al.*, 2022). No significant cross-reactivity was detected and this was determined through the investigation of 30 samples of patients who were suffering from parasitic or viral diseases. In line with this study, Liu et al (2005) also reported a sensitivity of 97.02 and 95.24%, for the detection of IgG and IgM antibodies against SARS-CoV-2. Specificity of their method for detecting both antibodies was reported to be 100% (Liu *et al.*, 2021).

The study done by (Liu *et al.*, 2021) revealed that serological assays are accurate and efficient methods for the screening for many pathogens, as specific IgM and IgG antibodies can be detected with enzyme-linked immunosorbent assay (ELISA), which has relatively high throughput capacity and less stringent specimen requirements. It was also reported in the study that N protein has high

immunogenic activity and is abundantly expressed. This is similar to other findings. This study used commercial recombinant N protein kits.

In addition to this, a study by Pei et al (2005) investigated the expression of N protein of SARS Coronavirus in bacterial and mammalian cells. In this study the N protein was expressed in *Escherichia coli* as a fusion with human glutathione transfers and confirmed by western blotting. There after the N protein was purified and then used to measure the SARS COV N specific antibodies in sera. According to this study, specific antibodies to SARS COV were 100% in SARS-CoV-2 patients' sera showing that this strategy could be used for SARS- CoV-2 sero-diagnosis (Pei *et al.*, 2005).

Interestingly, Djukic et al (2021) reported that for sensitive and reliable serological assay it is necessary to produce SARS-CoV-2 N antigens. Protein expression in prokaryotic systems, such as *E. coli*, is one cost effective way to rapidly provide high quantities of recombinant protein. In contrast to highly glycosylated S protein and its fragments, requiring eukaryotic expression. For instance, the N protein of SARS-CoV-1 have shown to be successfully expressed in *E. coli* (Chin *et al.*, 2021)

Several other groups have reported serological tests using the nucleocapsid protein from SARS-CoV-2 by Immunofluorescence assay.

Despite these findings, further studies are needed to understand antibody dynamics in persons infected with SARS-CoV-2 to determine the most sensitive and specific antibody assays and to use. Thus, a sensitive and specific antibody assay using heat-treated plasma/serum may enhance safety for those working with these fluids (Burbelo *et al.*, 2020).

COVID-19 serological tests are mainly based on detecting specific antibodies against SARS-CoV-2 antigens . As the virus enters in the human body it elicits an immune response to produce the antibody against the virus. Detection of such antibodies in infected persons is very useful whether the person has been exposed to the virus whether they have symptoms or not. The antibody detection test, however, is not recommended for the identification of active cases of SARS-CoV-2 infections (Prasad and Yadav, 2020a)

Many commercial companies and research institutes have developed and compared different serological tests to detect SARS-CoV-2 antibodies from patient serum or plasma samples. Currently, the common SARS-CoV-2 serological tests are available in ELISA and Lateral flow immune-chromatography methods. In both methods, the antibodies against one of the two main surface proteins of SARS-CoV-2 are detected (Nadoushan, 2020). The Center for Disease Control (CDC) has also developed a SARS-CoV-2 serological kit with a specificity of more than 99% and sensitivity of 96%, but still, this test does not have permission to be used as a diagnostic test (Centers for Disease Control and Prevention, 2022).

SCoV-2 *Detect*™ IgM ELISA kit and SCoV-2 *Detect*™ IgG ELISA kit (InBios International, Seattle, USA) is an in vitro, qualitative, indirect ELISA test used to detect the presence of IgM and IgG antibodies respectively against SARS-CoV-2 spike proteins (Kundu *et al.*, 2022).

SARS-CoV-2 Total (COV2T) and SARS-CoV-2 IgG (COV2G) are chemiluminescent immunoassays to detect the presence of total antibodies (IgM, IgG) and IgG, respectively in human serum and plasma. The assay uses the Spike-Receptor binding domain (S1-RBD) as antigen. A cut-off-index (COI) of 1 or more is considered positive (Kundu *et al.*, 2022)

Roche Elecsys® Anti-SARS-CoV-2 (Roche Diagnostics, Switzerland) is an electrochemilumiscent immunoassay for the detection of antibodies to SARS-CoV-2 nucleocapsid (N). The Abbott SARS-CoV-2 IgG (Abbott Diagnostics, IL, and USA) is also a CLIA for the qualitative detection of antibodies to the Nucleocapsid (N) protein of SARS-CoV-2. This assay is a qualitative detection of IgG antibodies against the SARS-CoV-2 in serum or plasma (Kundu *et al.*, 2022).

Kundu et al (2022) compared the performance of the above serological tests and found the sensitivity and specificity of *SARS CoV2 Detect IgG* ELISA to be 88.9% and 82.7%, respectively, compared to those of the CLIA Roche at 85.6% and 82%, respectively.

Another study by Kontou et al (2020) evaluated IgM and IgG tests based on Immunofluorescence assay (IFA) Enzyme-linked immunosorbent assay(ELISA), Chemiluminescence Immunoassays (CLIA), and the Lateral Flow Immunoassays (LFIA) (Kontou *et al.*, 2020). All methods yielded high specificity with some of the ELISA and LFIA reaching levels around 99%. ELISA and CLIA based methods performed better in terms of sensitivity (90%–94%) followed by LFIA and IFA with sensitivities ranging from 80% to 89%. ELISA tests could be a safer choice at this stage of the pandemic (Kontou *et al.*, 2020).

A clear advantage of serological tests over RT-PCR is that they can identify individuals previously infected by SARS-CoV-2, even if they never underwent testing while acutely ill (Sidiq *et al.*, 2020). Serological tests have generated substantial interest as an alternative or complement to RT-PCR and other Nucleic acid tests in the diagnosis of acute infection, as some might be cheaper and easier to implement at the point of care (Sidiq *et al.*, 2020).

Assays for detection of SARS-CoV-2-specific antibodies in serum or plasma can be used to make a retrospective diagnosis in individuals thereby helping policy makers make estimates of the proportion of population who have been infected by testing unbiased collection of sera in population-weighted serosurveys. In addition, serology assays are needed to establish the effectiveness and durability of immune responses to SARS-CoV-2 infection for correlating humoral immune responses with disease severity (Chansaenroj *et al.*, 2021) .

A study by Djukic, indicated that serosurveys are also very important for determining the real infection rate in a population. Apart from this, serosurveys are also important for identification of individuals who were infected but are asymptomatic, but in order to develop a reliable sero-assay, a suitable SARS- CoV-2 antigen is necessary (Djukic *et al.*, 2021).

2.6 Prevention and control

Unfortunately, there is no medication that has been approved and demonstrated an effect on COVID-19. The strongest and most effective weapon that society has is the prevention of the spread of the virus. Early screening, diagnosis and isolation are necessary to prevent further spread (Güner, Hasanoğlu and Aktaş, 2020).

Due to the continuous spread of the virus, long term effective control measures should be adopted. The main points in preventing the spread in society are hand hygiene, social distancing and quarantine. With increased testing capacity, detecting more COVID-19 positive patients in the community will also enable the reduction of secondary cases with stricter quarantine rules (Sharma, Farouk and Lal, 2021).

To reduce COVID-19 transmission from potentially asymptomatic or pre-symptomatic people, the ECDC recommends the use of face masks (WHO, 2020). The use of face masks in the community may primarily serve as a means of source control Studies have been conducted that support the

infectiousness of SARS-CoV-2 in the pre-symptomatic stage; social distancing is thus of critical importance in establishing control over the pandemic (WHO, 2020).

Several medications have been approved by the World Health Organization for relieving symptoms in mildly infected individuals. These include anti-viral medicines, paracetamol, and chloroquine (Agrahari *et al.*, 2021)

COVID-19 vaccines are now available on the market. The main types of vaccines available in Zambia are BioNTech Pfizer vaccine, Johnson and Johnson, Oxford AstraZeneca, Moderna and Sino pharm vaccine. Boosters are also recommended to maintain immunity. COVID-19 vaccines are effective at preventing infection, serious illnesses and so the risk of a vaccinated person experiencing the illness is reduced (Forni *et al.*, 2021).

This study aims at developing an in-house Immunofluorescence assay by using recombinant SARS-CoV-2 nucleocapsid protein as an antigen to detect antibodies in individuals that have been exposed to COVID-19. Currently there is no indigenous serological test which can be used for determining post-exposure to COVID-19. Developing and validating such an assay offers an alternative from commercial kits which may be costly.

Validation of this assay will be performed by determining the level of agreement between the in-house IFA and a commercially ELISA targeting the N gene using Cohens Kappa test. Sensitivity and specificity will be performed by comparing with a sufficient number of true positives and true negatives. The in-house IFA has the potential to be used for sero-surveillance studies to determine the true extent of the population exposed to COVID-19. This information is essential for assessing guidelines and effectiveness of control measures put in place by policy makers.

CHAPTER THREE

3.0 MATERIALS and METHODS

3.1 Study Design

This study was a laboratory-based study, focused on the development and performance evaluation of an in-house diagnostic test for SARS-CoV-2.

3.2 Preparation of competent cells

3.2.1 Preparation of calcium chloride buffer

Calcium chloride buffer was prepared using 5.5g anhydrous Calcium chloride ($CaCl_2$) by mixing with distilled water until all the $CaCl_2$ dissolved. The $CaCl_2$ solution was filter sterilized through a 0.22µm pore.

A working solution of $CaCl_2$ was prepared by mixing 10ml of $CaCl_2$ to 90mL distilled. The solution was then filter sterilized using 0.22µm filter sterilizing pores.

3.2.2 Culture of *E. coli* (DH5α)

E. coli (DH5α) stock solution was inoculated into 5ml ampicillin free Luria Bertani (LB) broth (Sigma-Aldrich, SA) and incubated overnight on a shaker at 37°C. There after 1ml of the *E. coli* was harvested and sub-cultured into 100ml fresh LB broth and incubated on a shaker at 37°C for 4 hours.

Calcium chloride working solution (0.1M) was chilled on ice. The *E. coli* culture was incubated on ice for 20 minutes. The culture was then centrifuged at 4000rpm for 10 minutes at 4 ºC and the supernatant was discarded. The pellet was resuspended in 20 ml ice cold $CaCl_2$ solution and incubated for another 20 minutes on ice and the resuspended cells were centrifuged at 4000rpm for 10 minutes at 4 ºC .

The collected cells were resuspended into 2.5 mL ice cold $CaCl_2$ containing 10% glycerol and aliquoted into Eppendorf tubes then stored at -80ºC until further use.

3.3 Preparation of recombinant SARS-CoV-2 N gene for cloning

Full SARS-CoV-2 virus genome material for amplification and cloning was derived from a sample stored at UNZAVET biobank. Viral RNA was extracted using QIAamp® Viral RNA Mini kit (250). The presence of SARS-CoV-2 virus was confirmed by real time RT-PCR using Maccura® SARS-CoV-2 detection kit which targets the N and E gene. Positivity was determined by analyzing the CT value of the curves on the light cycler. The cut off CT value was 25. The RT-PCR conditions were of 55°C for 15 minutes, 95°c for 2 minutes ,95°C for 15 seconds, 58°C for 35 seconds and finally 37°c for 30 seconds for 35 cycles.

3.3.1. cDNA Synthesis

SARS-CoV- 2 cDNA was synthesized from the RNA template using conventional RT-PCR. This was performed using 5 µl of extracted viral RNA added to 20µl of total reaction mixture using SuperScript IV Reverse Transcriptase (RT), containing 50 µM primers. The concentration of the cDNA was measured and the cDNA was stored at -80°C.

3.3.2 N gene amplification

SARS-CoV-2 N gene was amplified from the SARS-CoV-2 cDNA by PCR using N-gene primers tagged with *SacI* and *SphI* restriction enzymes recognition sites (Table 1).

Table 1: N gene primers and restriction enzymes

Primer name and restriction enzymes	**Primer sequence***
SARS-CoV2-N-*SacI* Forward	5'-ttc**GAGCTC**atgtctgataatggaccccaaaatcag-3'
SARS-CoV2-N-*SphI* Reverse	5'-cga**GCATGC**ttaggcctgagttgagacatcac-3'

*Uppercase letters are the restriction enzyme sites

The SARS-CoV-2 cDNA was used as a template for PCR by adding 5 µL of cDNA to 45µL of total reaction mixture containing 10X transcription buffer$_2$, 2µl of each primer, Q5 Hot Start High-Fidelity 2X Master Mix DNA polymerase 16 µl nuclease free water.

The master mix for the PCR was prepared using 10X master mix, 0.2µM SARS-CoV-SacI forward primer, 0.2µM SARS-CoV-SphI reverse primer, 16µL nuclease free water and 5µL of cDNA. A confirmed SARS-CoV-2 N gene was used as a positive control and nuclease free water was used as a negative control.

The PCR was run under the cycle conditions of 98°C for thirty seconds, 98°C for thirty seconds, 59°C for thirty seconds, 72°C for thirty seconds, for 35 cycles , 72°C for five minutes and finally 4°C for infinity.

The amplified products were visualized on 1% agarose gel stained with ethidium bromide and the expected band size was 1200bp.

3.4 Plasmid Vector Preparation

3.4.1 Plasmid Restriction Digestions

The multiple cloning sites of the pCAGGS plasmid and SARS-CoV-2 N gene were screened to ensure that the selected restriction enzymes had no targets within the N gene. The plasmid was digested using restriction enzymes *SacI* and *SphI.*

This was carried out by preparing a reaction mixture of 10X buffer, plasmid DNA, *SacI* and *SphI*. In the second reaction, only one restriction enzyme was included, *SacI,* and in the third reaction only *SphI* was included. A negative control constituting of 10X buffer, plasmid DNA and no restriction enzyme was also included as shown in Table 3 below:

Table 2: Reaction mixtures for pCAGGS digestion

	SacI	***SphI***
Reaction 1	✓	✓
Reaction 2	✓	×
Reaction 3	×	✓
Reaction 4	×	×

The reaction mixture was incubated for 1 hour. The plasmid digestion verification was performed using 1 % agarose gel electrophoresis.

3.4.2 Restriction digestion of Plasmid and SARS-CoV-N

pCAGGS-MCS and SARS-CoV-2 N gene having *SacI* and *SphI* ends was prepared for restriction enzyme digestion simultaneously in order to get corresponding ends in readiness for ligation. Master mix containing 10X buffer and nuclease free water was prepared, then 0.5µl *SacI* and 0.5µl *SphI* and 6µl plasmid DNA was added. The mixture was incubated for 1 hour at 37ºC.

SARS-CoV-2 DNA was also subjected to restriction enzyme digestion by adding 6µl of the SARS-CoV-2 DNA to a master mix containing 10X buffer, nuclease free water, 0.5µl *SacI* and 0.5 *Sph1*. The mixture was also incubated for 1 hour at 37ºC.

3.4.3 Ligation of the plasmid and N gene

The digested plasmid DNA was measured and the amount of DNA was found to be 56.18ng/µl. The N gene insert DNA was 30ng/µ*l*. A ligation reaction mixture was prepared as follows: Quick ligation buffer 5µl, QuickT4 ligase 2.5µl, plasmid 1µl, N gene insert 1µl and nuclease free water 15.5µl. The ligation mixture was incubated overnight at 20°C in a thermocycler.

3.4.4 Transformation of competent *E. coli (DH5α)*

The pCAGGS-SARS-CoV-N was transformed into the prepared competent *E. coli* cells as follows:

The frozen competent cells (-80ºC fridge) were thawed on ice. The ligation mix or plasmid was added to the competent cells and mixed gently. The mixture was incubated on ice for 10-15 minutes. The mixture was subjected to a heat shock by placing the reaction mix at 42°C for 60 seconds on a water bath. After the heat shock, the cells were transferred onto the ice and add 500uL of warm LB broth medium. The tubes were placed in the shaker (2000 rpm) at 37°C for 1 hour to stabilize the cells. After the incubation, the reaction mixture was given a brief spin at 4000 rpm for 2-3 minutes and the supernatant was discarded. The mixture was spread onto different plates with appropriate Ampicillin antibiotic selection. The plates were incubated overnight at 37°C.

After 12-16 hours of plating, colonies were observed on the plates and insert check/verification PCR for successful colonies was performed.

3.4.5 Insert Verification

3.4.5.1 Direct Colony PCR

Insert check was performed by direct colony PCR and by purified colony PCR. PCR was performed on selected colonies using vector specific primers pCAGGS forward and pCAGGS reverse (Table 3) in order to detect presence of the insert (Jafari *et al.*, 2022).

Table 3: pCAGGS primers

Primer name	**Primer sequence**
pCAGGS Fw (M13 Fw)	5’ ttccGAGCTccgaggaattcactcctcagg 3’
pCAGGS Rv (M13 Rv)	3’tgatGCATGCgagacagcacaataaccagca 5’

A total volume of 50µl solution consisting of 25µl master mix, 2µl pCAGGS forward primer ,2µl pCAGGS reverse, 20µl nuclease free water and 1µl DNA(colony) was used for the PCR under conditions of 94°C for 1 minute, 94°C for 30 seconds, 53°C for 30 seconds, 68°C for 2 minutes and finally 4°C for 35 cycles.

3.4.5.2 Plasmid Purification and insert check

The plasmid from the selected colony was purified using OREGENE PowerPrep™ HP Plasmid Purification maxiprep kit according to the manufacturer’s instructions. After purification PCR was performed using vector specific forward and reverse primers (Table 3) and the products were

analyzed using agarose gel to detect difference in the sizes of the bands. Complete pCAGGS-Multiple cloning sites (+) with no insert was used as a control.

3.4.5.3 Plasmid orientation check

Insert orientation verification was performed by use of insert specific and vector specific primers for PCR of the purified plasmid. The correct orientation of the insert was determined by distinguished size of bands according to the distance between the primers as seen in Figure 1 . The first primer set was pCAGGS Fw and insert specific primer SARS-CoV-2- 29082 Rv which are 800bp apart. The second set was pCAGGS Fw paired with SARS-CoV-2 -*SphI* Rv which is 1300 bp apart. The third set was pCAGGS Rv and SARS-CoV-2- *SacI* Fw which is 1300 bp apart and lastly SARS-CoV-2 29082 Fw and pCAGGS Rv.

Table 4: Primers used for insert orientation PCR

Primer name	**Primer sequence**
M13 Fw	5’ ttccGAGCTccgaggaattcactcctcagg 3’
M13 Rv	3’tgatGCATGCgagacagcacaataaccagca 5’
SARS-CoV-2-*SphI* Rv	5’cgaGCATGCttaggcctgagttgagacatcac 3’
SARS-CoV-2-*SacI* Fw	5’ttcGAGCTCatgtctgataatggaccccaaaatc 3’
SARS-CoV-2 29082 Fw	5’ cggtttaccttatcccaatggaccggtaacctga 3’
SARS-CoV-229082 Rv	5’ tagccttaaaacctcgaatcgaatgcctttgaac 3’

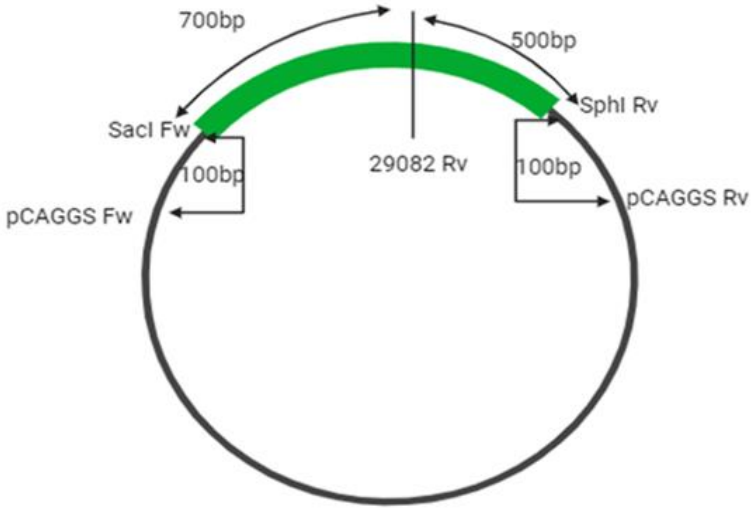

Figure 1: Location of the different primers on pCAGGS.

3.4.5.4 Sequencing of insert

Colonies were selected and prepared for sequencing in order to verify that the insert SARS-CoV-2 N gene was the correct nucleocapsid gene sequence with no mutations and cloned in the correct orientation. PCR was setup to amplify the pCAGGS-SARS-CoV-2 N gene.

The PCR products were transferred into Eppendorf tubes and 2 µl of 125mM EDTA was added to each Eppendorf tube and mixed. 90µl of 100% ethanol was added to the mixture was incubated at room temperature for two minutes. The mixture was centrifuged at 15000 rpm for 20 minutes at 4°C, supernatant removed followed by addition of 200µl 70% alcohol. Without mixing the samples were centrifuged at 15000 rpm for 10 minutes at 4°C.The supernatant was discarded and the samples were dried in a vacuum dryer while covering with aluminum foil. 20 µl HiDi formamide

was added to the tubes and vortexed. The resultant product was then analyzed using sanger sequencing method. After sequencing, each sequence was blasted in NCBI to confirm that it was SARS-CoV-2.

3.5 Transfection of SARS-CoV-2 Nucleocapsid protein in Vero E6 cells

3.5.1 Vero-E6 cell culture

Frozen Vero E6 cells were thawed followed by addition of DMEM containing 3 mL $NaHCO_3$, 5 mL L-glucose, 6ml D-glucose, 10% fetal bovine serum and 5 mL Pen-Strep. The cells were centrifuged and the supernatant was discarded. The pellet was resuspended in DMEM and incubated at 37°C and 5% CO_2. When the cells reached confluency, DMEM media was removed from the flask followed by washing with PBS twice. The PBS was removed and trypsin was added to the flask followed by incubation at 36.8°C for 3 minutes. After incubation 5ml of media was added and the cells were transferred to a conical flask and centrifuged at 2000rpm for 2 minutes. The supernatant was removed. Following this, 5ml of media was added to the culture flask and another 5ml media was added to the sediment in the conical flask and the cells were resuspended. Lastly 2mls of the resuspended cells were pipetted into the culture flask then incubated for 72 hours at 37ºC and 5% CO_2 hours until the cells became confluent.

3.5.2 pCAGGS -SARS-CoV-2 N gene Transfection

When the cells reached confluency, 2.5ml complete growth media was pipetted in a 6 well plate and then incubated overnight. Transfection reagent TransIT-LT1 was prepared and warmed to room temperature. 250µl of Opti-MEM® reduced serum medium was put in 6 sterile tubes then 2.5µl of purified plasmid DNA from each colony was added and mixed gently. RVF plasmid DNA was added to one well and a negative control was also included (RVF, NC).

7.5µl of TransIT-LT1 (Mirus Bio, USA) was added to the diluted DNA mixture and pipetted gently to mix. The mixture was incubated at room temperature for 15-30 minutes. After incubation the TransIT-LT1 reagent -DNA complex was added drop-wise to the different areas of the well. The culture vessel was gently rocked to distribute the TransIT-LT1 reagent -DNA complexes and incubated for 72 hours at 37 ºC and 5% CO_2.

3.6 Development of an in-house IFA

After 24 hours the cells were harvested and 20µL of cell suspension was seeded on 24- well IFA slides. Three wells were seeded with RVF transfected cells and three wells with nuclease free water.

The slides were fixed by first dipping them in PBS and then dipping in acetone for five minutes. After fixing, the cells were incubated at room temperature for one hour.

Archived serum samples (ten) from individuals previously infected with COVID-19 were obtained from the biobank. The Serum samples were tested using On-Site COVID-19 IgG/IgM Rapid test (CTK Biotech, USA) according to the manufacturer's instructions and found to be positive. The positive serum samples were heated at 65°C for 30 minutes. The serum was left to cool and after cooling the samples were diluted in the ratio 1:50 with 50% glycerol. Following this 20µL of serum (COVID-19 positive) was added to the wells on the seeded IFA slides. Of the 24 wells, 3 wells were seeded with RVF positive serum, and 3 wells nuclease free water. The IFA plates were incubated at 37°C for 1 hour at room temperature. The slides were washed twice in PBS and then flooded with conjugate antibody goat anti human IgG (1:500) followed by incubating at 37°C for 1 hour 30 minutes. The slide having RVF was flooded with RVF conjugate antibody. Lastly the slides were flooded with 50% glycerol, covered with a coverslip and viewed using a fluorescence

microscope. The recombinant SARS-CoV-2 N gene was later tested on SARS-CoV-2 monoclonal antibodies

For a positive IFA sample, it is expected that a bright green fluorescence light would be seen in the cytoplasm of the cells on the slide due to antigen antibody binding and for a negative IFA result no bright green fluorescence is expected.

The recombinant SARS-CoV-2 N antigen was tested on SARS-CoV-2 monoclonal antibodies. This was performed by seeding Vero-E6 on the IFA slides. 20µl of monoclonal antibody suspension was added to the wells on the seeded IFA slides. A positive control was included as well as a negative control where nuclease free water was used instead of monoclonal antibodies.

For validation of the IFA, the IFA results are supposed to be compared to a gold standard followed by determining the sensitivity and specificity of the assay.

All methods used for this study were followed according to standard procedures

Ethical clearance was obtained from ERES Converge and the request to conduct request research was granted by National Health Research Authority (NHRA) as shown in appendix II and III.

CHAPTER 4

4.0 RESULTS

4.1. Recombinant SARS-CoV-2 N gene preparation

The extracted SARS-CoV-2 viral nucleic acid was found to be positive and had a CT value of the curve of 25 (CT=25).

Amplification of the N gene using primers tagged with *SacI* and *SphI* showed an amplification of 1200 base pairs implying that the N gene was successfully amplified (Figure 2).

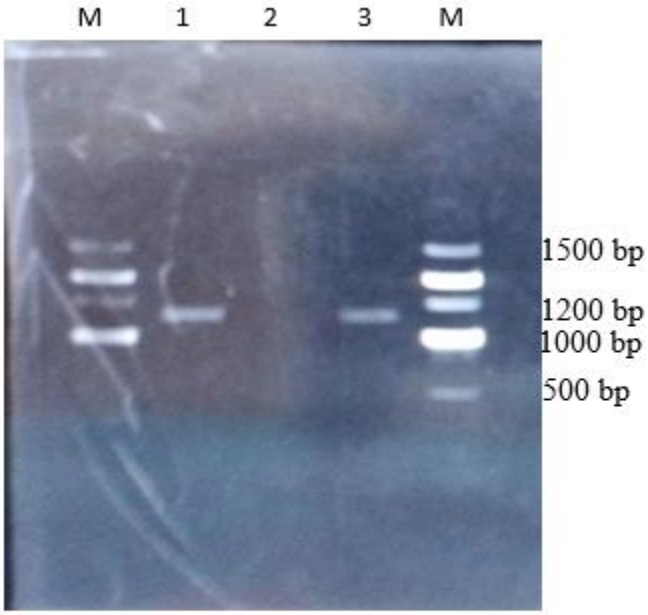

Figure 2: Agarose gel showing N gene amplification

Key : Lane M: marker, Lane 1: SARS-CoV-2 DNA plus N gene primers, Lane 2: Negative control (SARS-CoV-2 DNA plus nuclease free water), Lane 3: Positive control (SARS-CoV-2 DNA plus N gene primers), Lane M: Marker

4.2 pCAGGS-MCS (+) Digestion

Analysis of the agarose gel for vector digestion with *SacI* and *SphI* showed that the 3 reactions which had the restriction enzymes had a short migration distance 1300 bp compared to the reaction that had no enzyme (Figure 3).

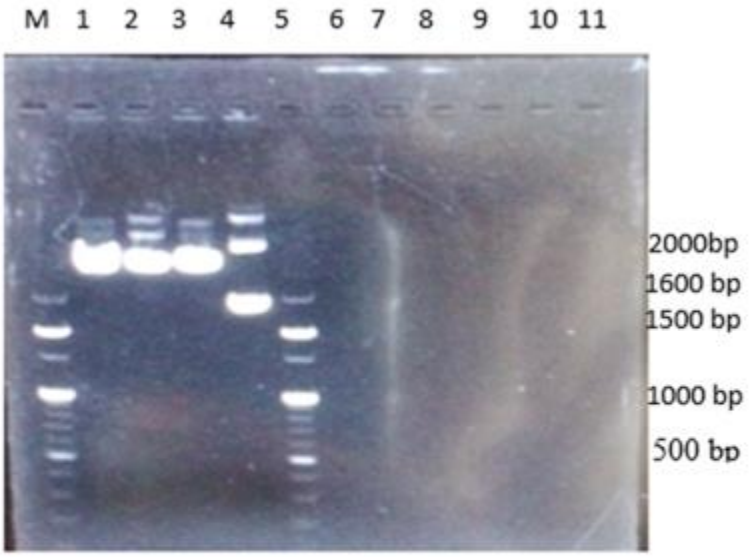

Figure 3: Restriction enzyme digestion of pCAGGS

Key: Lane M: marker, Lane 1:pCAGGS plus *SacI*, Lane 2: pCAGGS plus *SphI*, Lane 3: pCAGGS plus *SacI* and *SphI*, Lane 4: pCAGGS with no restriction enzymes.

4.3 Transformation of pCAGGS- N gene into *E.Coli*

Colonies were seen on the LB agar plate which was cultured with transformed *E.coli* (Figure 4).

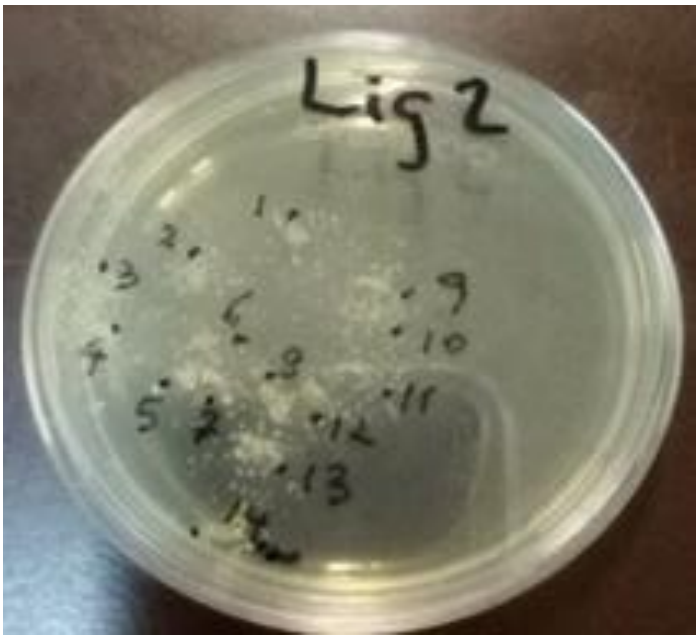

Figure 4: LB agar plate (+Amp) with E.coli transformed with pCAGGS-N gene.

4.4 Insert Verification

4.4.1 Direct Colony PCR

Insert check by direct colony PCR showed that some colonies had the correct insert. PCR products in lane 6,8,11,13 showed that they had the correct insert of 1200 bp (Figure 5).

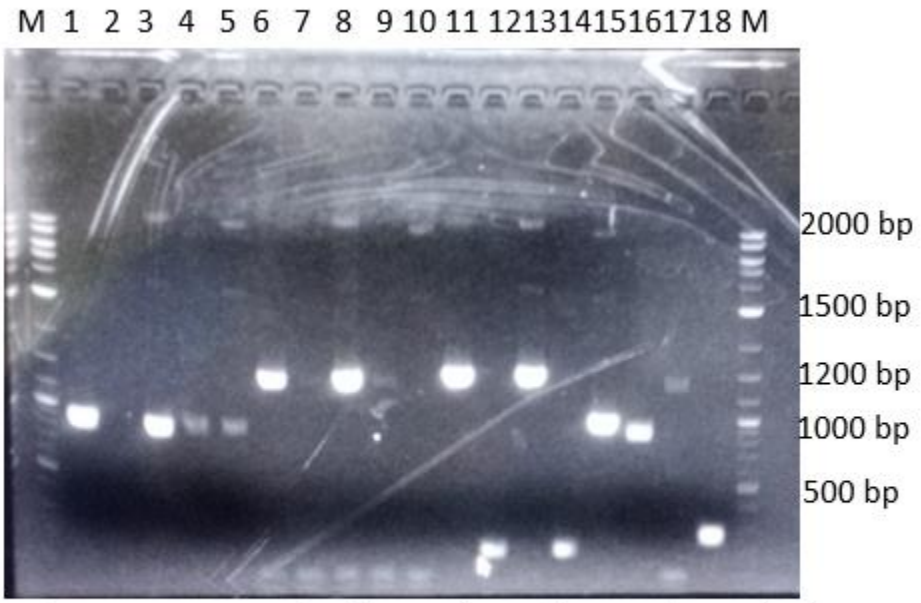

Figure 5: Agarose gel with amplicons for PCR insert check
Key: Lane M: marker, Lane 2: Negative control , Lane (3,4,5,15,17,18): PCR amplicon, Lane (6,8,11,13): PCR insert amplicon 1200bp.

4.4.2 Purified plasmid insert check

Purified colony PCR products were visualized on agarose gel and showed that some colonies had the correct insert of 1200bp (Figure 6). Colonies that migrated 1200bp compared to pCAGGS show that the plasmid had the correct insert. Colonies with migration same as pCAGGS or more show that the insert size was not correct.

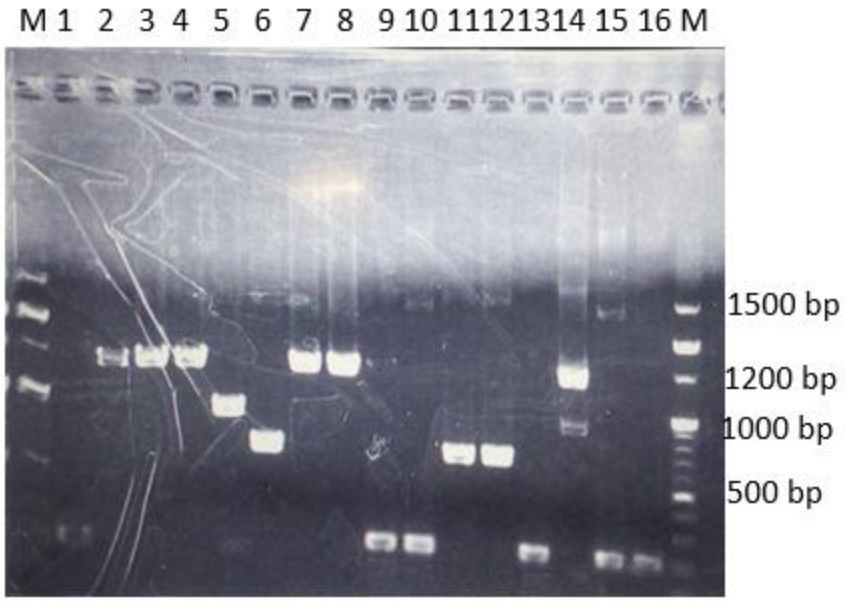

Figure 6: Agarose gel showing purified colonies PCR amplicons for insert check
Key: Lane 2: Negative control, Lane (3-5,8-11,14-17): Amplicon with incorrect insert, Lane (7,12,13): 1200 bp amplicon.

Direct colony PCR and purified colony PCR agarose gels had amplicons of 1200bp which showed the insert was actually present.

4.4.3 Insert orientation

Results for insert orientation were determined by the distinguished size of bands as seen on the agarose gel (Figure 7).

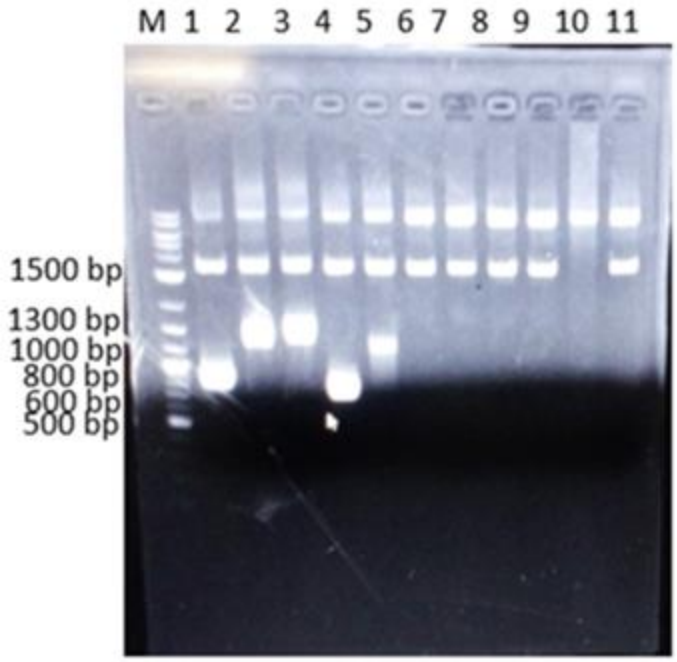

Figure 7: Colony amplicons to determine insert orientation.

Key: Lane M: marker, Lane 1: pCAGGS Fw plus 29082 Rv, Lane 2: pCAGGS Fw plus SARS-CoV-2 *SphI* Rv, Lane 3: pCAGGS Rv plus SARS-CoV-2 *SacI* Fw, Lane 4: 29082 Fw plus pCAGGS Rv.

Lane 1 used primer set pCAGGS Fw and 29082 Rv and gave an amplification of 800bp. Lane 2 used primer sets pCAGGS Fw-SARS-CoV-2 *SphI* Rv and gave an amplification of 1300bp, lane 3 used pCAGGS Rv and *SacI* forward giving 1300bp, lane 4 used primer set 29082 Fw and pCAGGS Rv and gave an amplification of 600bp. The distance between each of the primer sets used was correct which showed that the insert was in the correct orientation.

4.4.4 Sequencing of insert

4.4.4.1 Colony DNA purification for sequencing

The DNA from all the colonies confirmed to have the recombinant N gene was run on a gel (Figure 8). The purified DNA was measured before sequencing (Table 5)

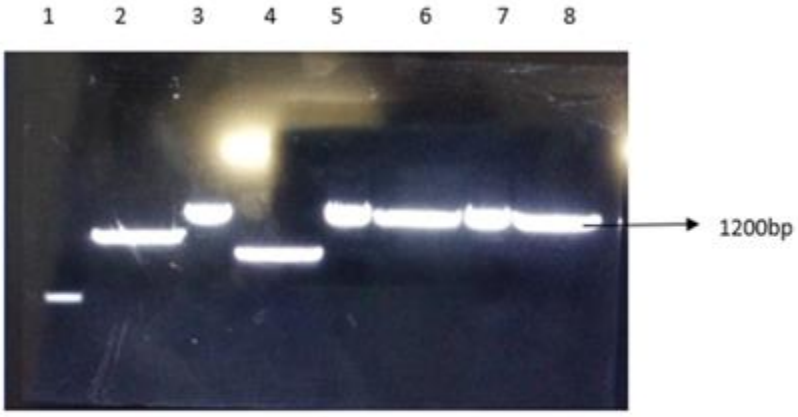

Figure 8: Colony PCR in readiness for sequencing
Key: Lane 1-pCAGGS MCS, Lane 2-pCAGGS-RVF-N, Lane 3-Colony S, Lane 4 -colony 10, Lane 5-Colony 1, Lane 6-Colony 11, Lane 7- colony 6, Lane 8-Colony S.

Lane 4 having pCAGGS-SARS-CoV-2 N gene from colony 10 had a faster migration which showed that the insert may not be present hence it was not sequenced.

Table 5: Purified DNA concentration

Colony	**1**	**6**	**11**	**S**	**RVF**
DNA µL	45.53	39.18	38.76	93.36	40.74

DNA concentration of SARS-CoV-2 N gene from colony 1,6,11,S and RVF N gene.

4.4.4.2 Sequencing results

The purified plasmid from the four colonies which were sequenced and blasted in National Center for Biotechnology Information(NCBI) showed that colony S had a similarity of 99.92% with SARS-CoV-2 N gene accession number OP016862.1 and E value of 0.0. Colony C1, C6 and C11 had 99.92% similarity with SARS-CoV-2 N gene accession number MZ393809.1 and E values of 0.0 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequenced colonies were aligned in GeneDoc using SARS-CoV-2 reference sequence accession number OR758451.1 as shown in the aligned sequences in appendix I (

The colonies that were sequenced showed that they were all SARS-CoV-2 N gene full sequences and that the proteins are complete meaning coding is complete, because there were no stop codons within the gene as seen in appendix II (https://genedoc.software.informer.com/download/).

4.5 Immunofluorescence assay (IFA)

The IFA plates that were transfected with pCAGGS -SARS-CoV- N antigen showed no fluorescence on all the wells having COVID-19 positive serum. The positive control pCAGGS-RVF was seeded with RVF positive serum and showed fluorescence while the negative control showed no fluorescence.

The recombinant SARS-CoV-2 N when tested on monoclonal antibodies showed that the antigen was successfully expressed despite the non-reactivity to human serum from exposed individuals.

Validation

Validation of the in-house IFA could not be performed because the IFA results were negative.

CHAPTER FIVE

5.0 DISCUSSION

The rapid spread of SARS-CoV-2, the etiological agent for COVID-19, has posed a huge threat to public health worldwide. RT-PCR is a common routine strategy for COVID-19 diagnosis but it can only be used for acute COVID-19 diagnosis and not post-exposure purposes. Therefore, there is need to develop serological tests that can detect presence of antibodies because only a trace of pathogen is enough to induce a human humoral response (Liu *et al.*, 2021). Furthermore, it is necessary to develop serological assay for sero-surveillance studies because they can be used to determine the exposure status of individuals in a population.

In this study an in-house immunofluorescence assay was designed to detect SARS-CoV-2 antibodies using recombinant Nucleocapsid protein, a highly immunogenic component in the virion, as an antigen. The significant findings from this study suggests that SARS-CoV-2 nucleocapsid gene can be cloned and expressed in a mammalian expression system. The purpose of protein expression was to generate the SARS-CoV-2 antigen which would be used to screen human serum samples for SARS-CoV-2 antibodies. It was confirmed that protein expression had successfully occurred by testing with monoclonal antibodies which had a positive result. Surprisingly it was found that none of the COVID-19 positive serum gave a positive reaction on the assay.

SARS-CoV-2 nucleic acid was obtained and confirmed to be positive by RT-PCR. Further, PCR targeting the N gene was performed on the SARS-CoV-2 nucleic acid and the results showed an amplification of 1200bp. This implies that the SARS-CoV-2 N gene which was the gene of interest for this study was successfully amplified. This finding concurs with a finding from a study by

Amine (2020) who in their study successfully obtained the SARS-CoV-2 N gene and ligated it into a plasmid.

Plasmid digestion by *SacI* and *SphI* showed that the plasmid having restriction enzymes migrated a shorter distance compared to the plasmid with no restriction enzymes. This showed that the plasmid was successfully digested. The transformed *E.coli* DH5 α cells were cultured for propagation on LB agar . Since the plasmid had an ampicillin resistant gene, the presence of colonies on the LB agar plate having ampicillin meant that that transformation was successful. These results agree with previous studies (Ranjbar *et al.*, 2022).

Confirmation of the presence of the insert (SARS-CoV-2 N gene) on the plasmid was performed using direct colony PCR and amplicon of 1200 bp was seen which is the size of the N gene. The presence of the insert was further proved by purified colony PCR and the findings showed some amplicons having a band size of 1200bp while others showed band sizes more or less than 1200bp. This means that the amplicons with a band size of 1200bp had the correct insert and the ones with less or more had an incorrect insert. The reason for these unexpected results could be that some of the plasmids did not ligate with the insert. Another possible reason could be that some of the DH5α cells were not transformed with the plasmid.

Confirmation that the insert was the correct SARS-CoV-2 sequence was performed by sequencing the recombinant SARS-CoV-2 N gene, blasting in NCBI and comparing with a SARS-CoV-2 reference sequence. This study found that the sequences were all similar to the SARS-CoV-2 N gene reference sequences. This result was significant because it showed that the SARS-CoV-2 recombinant N gene used in transfection of Vero E6 cells for protein expression was correct. Twenty-four hours post transfection, the cells were plated on IFA plates and tested them using

COVID-19 positive serum and it was found that the COVID-19 positive serum was non-reactive to the recombinant antigen.

Contrary to the current study, a similar previous study by Amine et al (2020), used prokaryotic expression system (*E.coli)* to express the nucleocapsid protein. The reason could be that they used *E.coli* for high level production of SARS-CoV-2 N protein. For this study eukaryotic cells were used so the protein expression occurs in a manner mimicking that of mammalian cells. Additionally, mammalian expression system is advantageous in maintaining the correct protein (García-Cordero *et al.,* 2021).

Despite confirmation that the cells had expressed the antigen, there was no reactivity of the antigen to the serum. The possible reason for negative IFA could be that the COVID-19 serum had a low reactivity to the recombinant protein or the protein expressed being non-reactive. Negative results are just as important as positive results in research because they can help alter or redesign the experiments in order to get the desired results. In this study the N protein was expressed, but it could be that it was not reactive when tested with polyclonal serum. Perhaps altering the expression system by using virus-like particles would lead us to positive results. Unlike using isolated single protein or natural virus isolates used in this study, Virus-like particles comprising of the nucleocapsid protein, spike protein and membrane protein could be a better option. This is because there is a greater likelihood that the antigen will maintain its natural conformation and overall, there could be a greater number of antigens available (Hirschberg *et al.,* 2022).

This study was limited in that the time frame from when the individuals were infected and when the serum was collected was not known. This might have affected the IFA results because most studies report that it takes 1-3 weeks after infection for antibodies to show up in the blood (Kohmer

et al., 2020). In this study the sampling time and how long the samples had been stored was not known.

Other studies have described different strategies for producing recombinant antigens, such as full-length S1, RBD, or N of SARS-CoV-2 from various eukaryotic expression systems, and prokaryotic cells, such as *E. coli*, for their potential production and have used them to develop inhouse serology tests, making them available options (Liu *et al.*, 2021),(Michel *et al.*, 2020),(Nguyen, Skelly and Goonawardane, 2021).

Recombinant protein-based immunoassays have been used to detect antibodies against different viruses such as rift valley fever virus (Kajihara, Dautu and Saasa, 2018), swine vesicular disease or human papilloma virus (Du, 2015). In the case of SARS-CoV-2, commercial lamina flow assays are available and a study by Hirschberg (Hirschberg *et al.*, 2022) used virus-like particles to detect antibodies against SARS-CoV-2. This is the first study which attempted to use an isolated N protein to detect antibodies to SARS-CoV-2 using IFA.

Most studies that have used recombinant N protein to detect antibodies to SARS-CoV-2 in general have used ELISA with virus-like particles (VLP) to achieve this purpose and the sensitivity and specificity of the assays have been measured.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In this study, recombinant SARS-CoV-2 N protein was produced and an in-house IFA test was developed. However, the house IFA test could not be validated. The reason for not validating the IFA could be because of the expression system used which produced a less reactive protein or the serum samples having low positivity. The results of this study showed that SARS-CoV-2 just like the previous SARS-CoV and MERS-CoV can be cloned into a plasmid, transformed into *E-coli* and transfected into mammalian Vero E6 cells. The recombinant SARS-CoV-2 N gene was correct and in the right orientation with no evidence of mutations observed after sequencing.

The expression and purification of recombinant SARS-CoV-2 N protein was successfully achieved in Vero E6 cells. Protein expression was also verified using monoclonal antibodies. Despite this, the IFA could not detect SARS-CoV-2 antibodies in human serum samples.

Currently there is no in-house serological assay for detection of antibodies to SARS-CoV-2 in Zambia. Therefore, the successful production of recombinant proteins entails that this strategy can potentially be used for development of serological diagnostic tools for sero-surveys or serological assays, however altering the system by using VLPs could give us desired results so that the system is validated.

6.2 Recommendation

An ELISA system using Virus-like Particles (VLPs) would be an option because VLPs have shown to have higher reactivity to polyclonal serum compared to recombinant proteins. For instance, Liu et al (2021), successfully expressed and applied recombinant N gene protein to develop an ELISA (Liu *et al.*, 2021). VLPs comprising of S, N and E gene have been used to successfully develop an ELISA (Xu *et al.*, 2020). Another option would be to obtain serum from individuals who are 1-3 weeks post infection with the virus.

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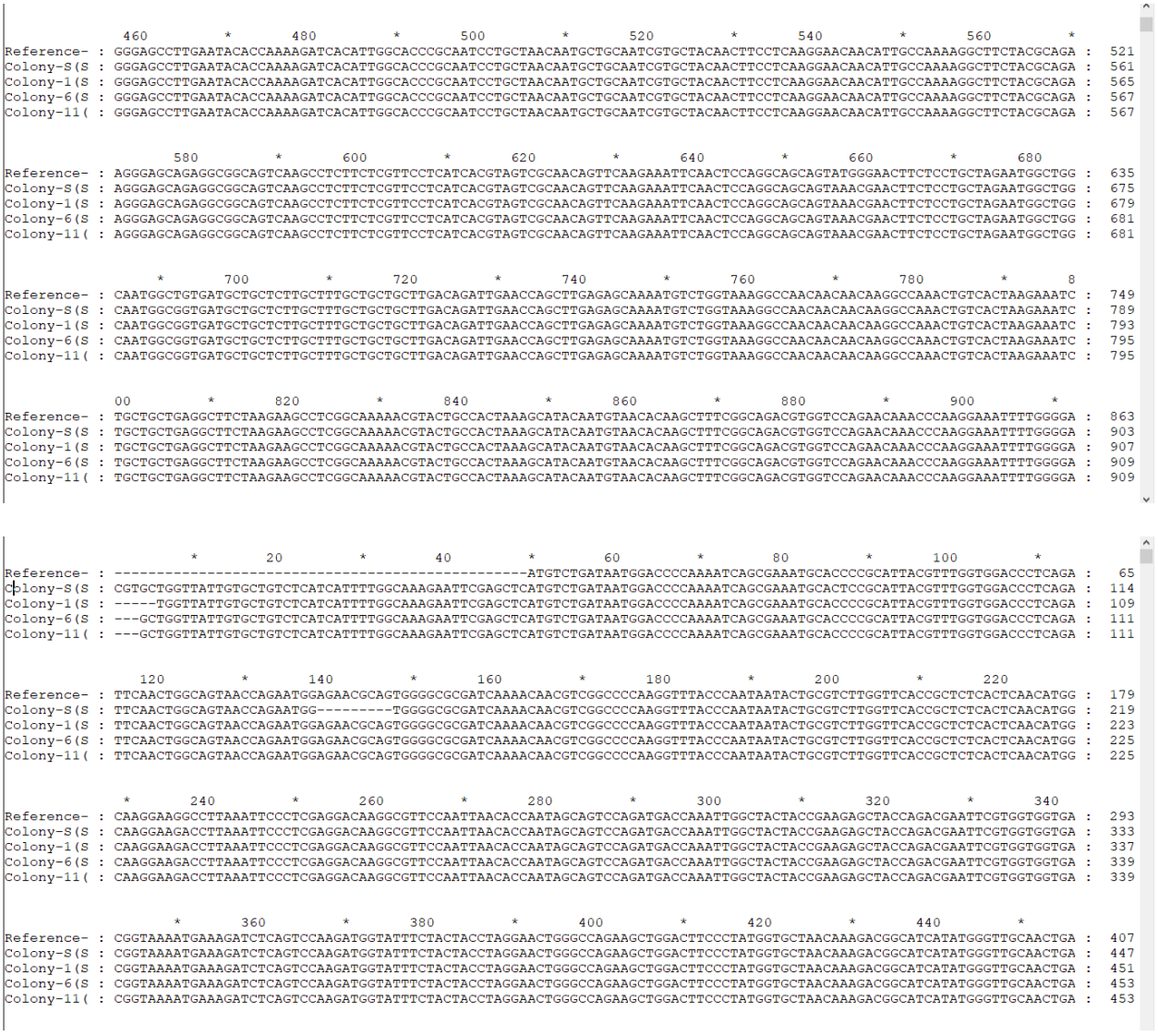
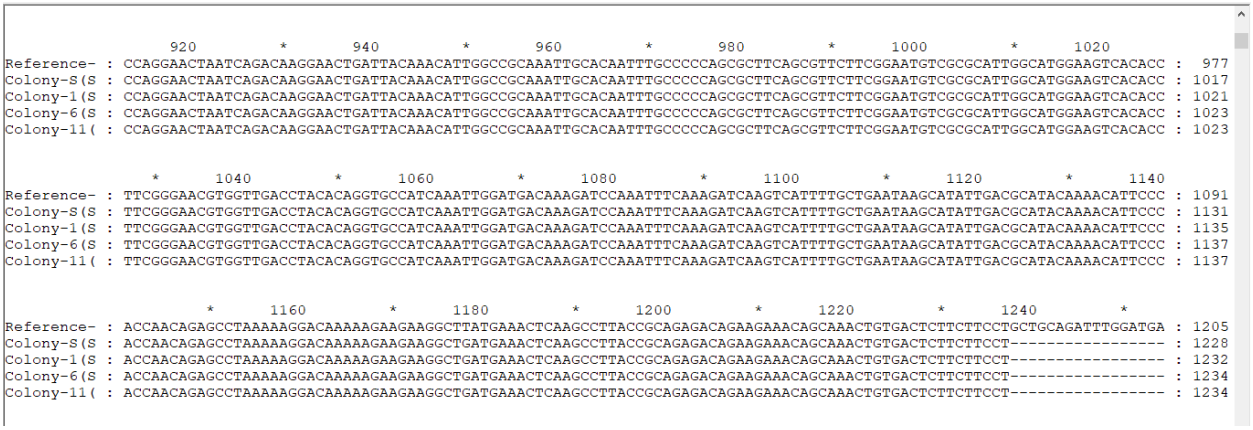
APPENDICES

Appendix I: Recombinant SARS-CoV-2 Sequence



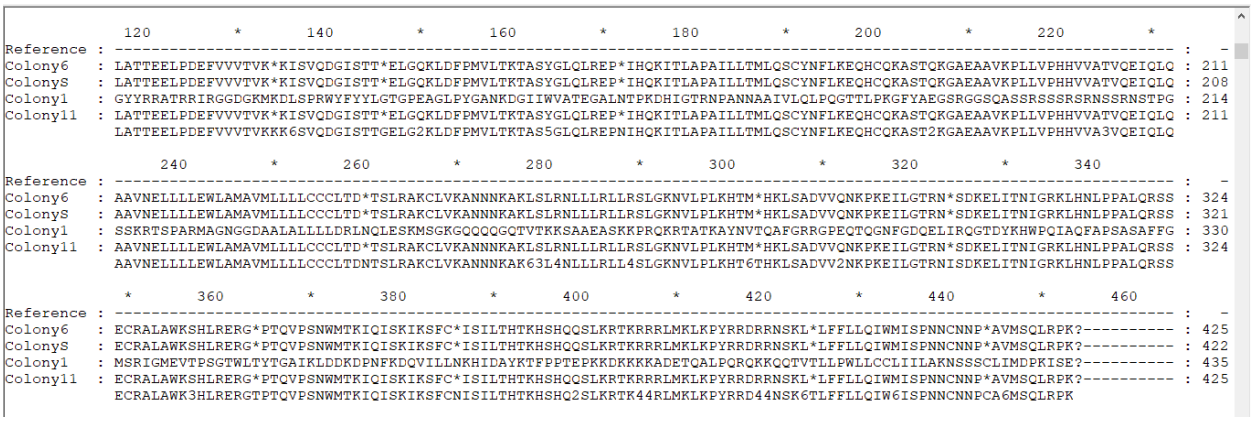


```
                920         *       940         *       960         *       980         *      1000         *      1020
Reference- : CCAGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACC :  977
Colony-S(S : CCAGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACC : 1017
Colony-1(S : CCAGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACC : 1021
Colony-6(S : CCAGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACC : 1023
Colony-11( : CCAGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACC : 1023

              *      1040         *      1060         *      1080         *      1100         *      1120         *      1140
Reference- : TTCGGGAACGTGGTTGACCTACACAGGTGCCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATTGACGCATACAAAACATTCCC : 1091
Colony-S(S : TTCGGGAACGTGGTTGACCTACACAGGTGCCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATTGACGCATACAAAACATTCCC : 1131
Colony-1(S : TTCGGGAACGTGGTTGACCTACACAGGTGCCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATTGACGCATACAAAACATTCCC : 1135
Colony-6(S : TTCGGGAACGTGGTTGACCTACACAGGTGCCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATTGACGCATACAAAACATTCCC : 1137
Colony-11( : TTCGGGAACGTGGTTGACCTACACAGGTGCCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATTGACGCATACAAAACATTCCC : 1137

                    *      1160         *      1180         *      1200         *      1220         *      1240         *
Reference- : ACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTTATGAAACTCAAGCCTTACCGCAGAGACAGAAGAAACAGCAAACTGTGACTCTTCTTCCTGCTGCAGATTTGGATGA : 1205
Colony-S(S : ACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGATGAAACTCAAGCCTTACCGCAGAGACAGAAGAAACAGCAAACTGTGACTCTTCTTCCT---------------- : 1228
Colony-1(S : ACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGATGAAACTCAAGCCTTACCGCAGAGACAGAAGAAACAGCAAACTGTGACTCTTCTTCCT---------------- : 1232
Colony-6(S : ACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGATGAAACTCAAGCCTTACCGCAGAGACAGAAGAAACAGCAAACTGTGACTCTTCTTCCT---------------- : 1234
Colony-11( : ACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGATGAAACTCAAGCCTTACCGCAGAGACAGAAGAAACAGCAAACTGTGACTCTTCTTCCT---------------- : 1234
```

Appendix II: Translated Protein

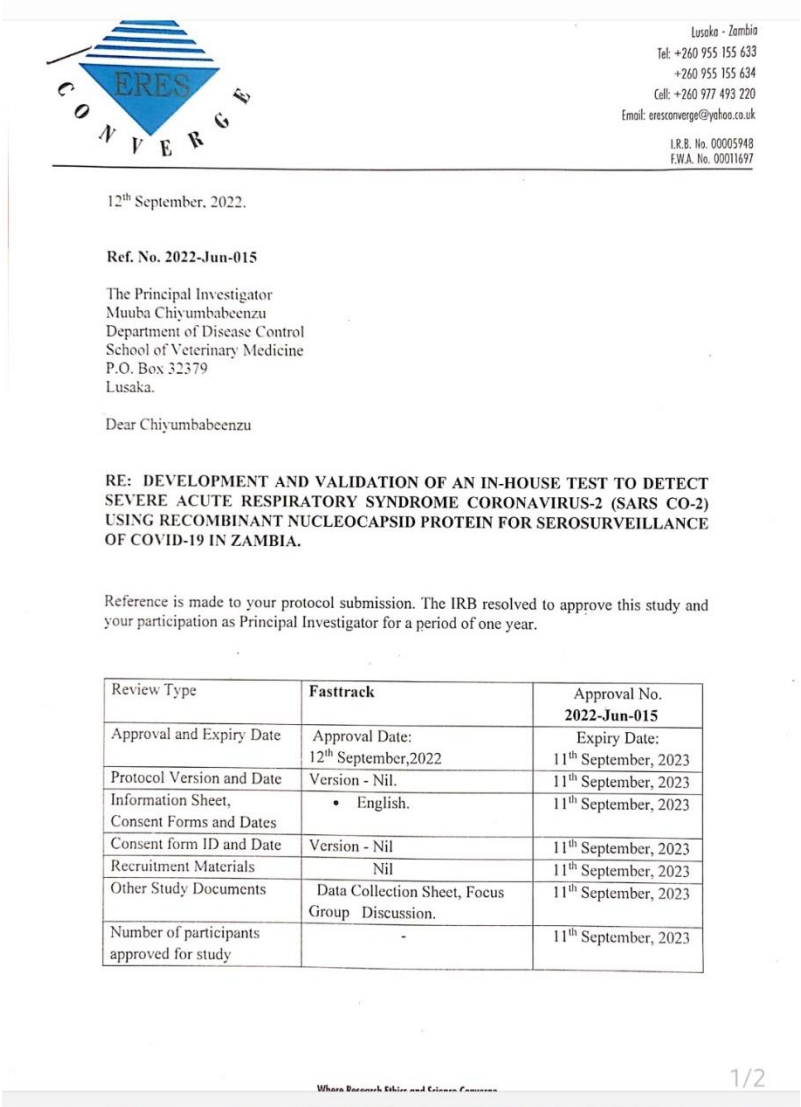


```
             120         *       140         *       160         *       180         *       200         *       220         *
Reference : ------------------------------------------------------------------------------------------------------------------------ :   -
Colony6   : LATTEELPDEFVVVTVK*KISVQDGISTT*ELGQKLDFPMVLTKTASYGLQLREP*IHQKITLAPAILLTMLQSCYNFLKEQHCQKASTQKGAEAAVKPLLVPHHVVATVQEIQLQ : 211
ColonyS   : LATTEELPDEFVVVTVK*KISVQDGISTT*ELGQKLDFPMVLTKTASYGLQLREP*IHQKITLAPAILLTMLQSCYNFLKEQHCQKASTQKGAEAAVKPLLVPHHVVATVQEIQLQ : 208
Colony1   : GYYRRATRRIRGGDGKMKDLSPRWYFYYLGTGPEAGLPYGANKDGIIWVATEGALNTPKDHIGTRNPANNAAIVLQLPQGTTLPKGFYAEGSRGGSQASSRSSSSRSRNSSRNSTPG : 214
Colony11  : LATTEELPDEFVVVTVK*KISVQDGISTT*ELGQKLDFPMVLTKTASYGLQLREP*IHQKITLAPAILLTMLQSCYNFLKEQHCQKASTQKGAEAAVKPLLVPHHVVATVQEIQLQ : 211
            LATTEELPDEFVVVTVKKK6SVQDGISTTGELG2KLDFPMVLTKTAS5GLQLREPNIHQKITLAPAILLTMLQSCYNFLKEQHCQKAST2KGAEAAVKPLLVPHHVVA3VQEIQLQ

                 240         *       260         *       280         *       300         *       320         *       340
Reference : ------------------------------------------------------------------------------------------------------------------------ :   -
Colony6   : AAVNELLLLEWLAMAVMLLLLCCCLTD*TSLRAKCLVKANNNKAKLSLRNLLLRLLRSLGKNVLPLKHTM*HKLSADVVQNKPKEILGTRN*SDKELITNIGRKLHNLPPALQRSS : 324
ColonyS   : AAVNELLLLEWLAMAVMLLLLCCCLTD*TSLRAKCLVKANNNKAKLSLRNLLLRLLRSLGKNVLPLKHTM*HKLSADVVQNKPKEILGTRN*SDKELITNIGRKLHNLPPALQRSS : 321
Colony1   : SSKRTSPARMAGNGGDAALALLLLDRLNQLESKMSGKGQQQQGQTVTKKSAAEASKKPRQKRTATKAYNVTQAFGRRGPEQTQGNFGDQELIRQGTDYKHWPQIAQFAPSASAFFG : 330
Colony11  : AAVNELLLLEWLAMAVMLLLLCCCLTD*TSLRAKCLVKANNNKAKLSLRNLLLRLLRSLGKNVLPLKHTM*HKLSADVVQNKPKEILGTRN*SDKELITNIGRKLHNLPPALQRSS : 324
            AAVNELLLLEWLAMAVMLLLLCCCLTDNTSLRAKCLVKANNNKAK63L4NLLLRLL4SLGKNVLPLKHT6THKLSADVV2NKPKEILGTRNISDKELITNIGRKLHNLPPALQRSS

             *       360         *       380         *       400         *       420         *       440         *       460
Reference : ------------------------------------------------------------------------------------------------------------------------ :   -
Colony6   : ECRALAWKSHLRERG*PTQVPSNWMTKIQISKIKSFC*ISILTHTKHSHQQSLKRTKRRRLMKLKPYRRDRRNSKL*LFFLLQIWMISPNNCNNP*AVMSQLRPK?---------- : 425
ColonyS   : ECRALAWKSHLRERG*PTQVPSNWMTKIQISKIKSFC*ISILTHTKHSHQQSLKRTKRRRLMKLKPYRRDRRNSKL*LFFLLQIWMISPNNCNNP*AVMSQLRPK?---------- : 422
Colony1   : MSRIGMEVTPSGTWLTYTGAIKLDDKDPNFKDQVILLNKHIDAYKTFPPTEPKKDKKKADETQALPQRQKKQQTVTLLPWLLCCLIILAKNSSSCLIMDPKISE?---------- : 435
Colony11  : ECRALAWKSHLRERG*PTQVPSNWMTKIQISKIKSFC*ISILTHTKHSHQQSLKRTKRRRLMKLKPYRRDRRNSKL*LFFLLQIWMISPNNCNNP*AVMSQLRPK?---------- : 425
            ECRALAWK3HLRERGTPTQVPSNWMTKIQISKIKSFCNISILTHTKHSHQ2SLKRTK44RLMKLKPYRRD44NSK6TLFFLLQIW6ISPNNCNNPCA6MSQLRPK
```

Appendix III: Ethical Clearance



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I.R.B. No. 00005948
F.W.A. No. 00011697

12th September, 2022.

Ref. No. 2022-Jun-015

The Principal Investigator
Muuba Chiyumbabeenzu
Department of Disease Control
School of Veterinary Medicine
P.O. Box 32379
Lusaka.

Dear Chiyumbabeenzu

RE: DEVELOPMENT AND VALIDATION OF AN IN-HOUSE TEST TO DETECT SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS-2 (SARS CO-2) USING RECOMBINANT NUCLEOCAPSID PROTEIN FOR SEROSURVEILLANCE OF COVID-19 IN ZAMBIA.

Reference is made to your protocol submission. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	**Fasttrack**	Approval No. **2022-Jun-015**
Approval and Expiry Date	Approval Date: 12th September,2022	Expiry Date: 11th September, 2023
Protocol Version and Date	Version - Nil.	11th September, 2023
Information Sheet, Consent Forms and Dates	• English.	11th September, 2023
Consent form ID and Date	Version - Nil	11th September, 2023
Recruitment Materials	Nil	11th September, 2023
Other Study Documents	Data Collection Sheet, Focus Group Discussion.	11th September, 2023
Number of participants approved for study	-	11th September, 2023

1/2

Where Research Ethics and Science Converge

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- A reprint of this letter shall be done at a fee.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB

Appendix IV: Ethical Clearance ; Authority to conduct research

NATIONAL HEALTH RESEARCH AUTHORITY

Lot No. 18961/M, off Kasama Road, Chalala, P.O. Box 30075, LUSAKA

Tell: +260211 250309 | Email: znhrasec@nhra.org.zm | www.nhra.org.zm

Ref No: NHRA1001/28/02/2024 **Date:6th March, 2024**

The Principal Investigator,
Muuba Chiyumbabeenzu,
University of Zambia,
School of Veterinary Medicine,
Lusaka, Zambia.

Dear Ms Chiyumbabeenzu,

Re: Request for Authority to Conduct Research

The National Health Research Authority is in receipt of your request for authority to conduct research titled **"Development and Validation of an In-House Serological Test to Detect Severe Acute Respiratory Syndrome Coronavirus-2 (Sars Cov-2) Using Recombinant Nucleocapsid Protein."**

I wish to inform you that following submission of your request to the Authority, our review of the same and in view of the ethical clearance, this study has been **approved** on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA bi-annually from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, University leadership, and all key respondents.

Yours sincerely,

Prof Victor Chalwe
Acting Director/Chief Executive Officer
National Health Research Authority