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SARS-CoV-2 MOLECULAR EVOLUTION AND HUMAN IMMUNE RESPONSE TO INFECTION

313.02. MEDICAL MICROBIOLOGY, VIROLOGY

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313.02 MICROBIOLOGIE, VIRUSOLOGIE MEDICALĂ

Teză de doctor în științe medicale

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The thesis was elaborated within the Departament of Preventive Medicine, Discipline of microbiology and immunology of ”Nicolae Testemițanu” State University of Medicine and Pharmacy, Republic of Moldova and Molecular Virology Laboratory, ICGEB, Trieste, Italia

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LIST OF ABBREVIATIONS

ACE2 - Angiotensin-converting enzyme 2

ADE - Antibody-Dependent Enhancement

ADCP - antibody-dependent cellular phagocytosis

ATP- adenosine triphosphate

BSL-3 - biosafety level 3

BSL-2 - biosafety level 2

CD - connected domain

CH - central helix

COVID-19 - Coronavirus-19 Disease

CoVs – Coronaviruses

CP - Convalescent Plasma

CT - cytoplasmatic tail

Ct - threshold cycle

CTD - C-terminal domain

D - aspartic acid

E – Envelope

ELISA- Enzyme-Linked Immunosorbent Assay

ER - endoplasmic reticulum

FDA - food and drug administration

FPs - fusion peptides

FPPR - fusion peptide proximal region

FRNT - Focus Reduction Neutralisation Test

G - glycine

GISAID - Global Initiative on Sharing All Influenza Data

HIV-1 - human immunodeficiency virus type 1

HLA - human leukocyte antigen

HR1 - heptad repeat 1

HR2 - heptad repeat 2

ICGEB - International Centre for Genetic Engineering and Biotechnology

IFN-γ - interferon-gamma

IgA - secretory immunoglobulin A

IgG - Immunoglobulin G

IgM - immunoglobulin M

kb - kilobase

LVs - lentiviral vectors

M – Membrane

MERS-CoV - Middle East respiratory syndrome

mRNA - messenger ribonucleic acid

N - Nucleocapsid

nAbs - neutralising antibodies

NSPs - nonstructural proteins

NTD - N-terminal domain

ORF - open reading frames

PRNT - plaque reduction neutralisation assay

RBD - Receptor Binding Domain

RBM - receptor-binding motif

RNA - ribonucleic acid

RNP - ribonucleoprotein

ROs - replication organelles

RT - reverse transcriptase

RTC - replication-transcription complex

RT PCR - reverse-transcriptase polymerase chain reaction

TM - transmembrane domain

TMD - transmembrane domain

TMPRSS2 - transmembrane protease serine 2

TRSs - Transcription Regulated Sequences

S – Spike

SARS-CoV

SARS-CoV-2 - Severe Acute Respiratory Syndrome Coronavirus 2

SD - subdomain

sgRNAs - subgenomic RNAs

SNP - single nucleotide polymorphism

SS - signal peptide

sVNT - surrogate viral neutralisation tests

VN - virus-neutralising

VOC - Variants of Concern

VOI - Variant of Interest

VSV - Vesicular stomatitis virus

VUM - Variants under Monitoring

UTR - untranslated regions

WHO - World Health Organization

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INTRODUCTION

Actuality and importance of the researched problem

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China, in December 2019 and quickly spread globally, causing the COVID-19 pandemic [1]. As of March 11th, 2023, the pandemic has caused over 760.4 million confirmed cases and 6,8 million deaths globally [2]. Since the outbreak, the virus has undergone rapid evolution, leading to the emergence of several variants of concern (VOCs) that are more transmissible, virulent, and potentially resistant to immunity induced by natural infection or vaccination. Up to date, the following VOCs were detected: alpha (B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2) and omicron (B.1.1.529, BA.2, BA.4, BA.5) [3,4], the first variants have been de-escalated as no more circulating.

Understanding the molecular evolution of SARS-CoV-2 and the human immune response to infection is critical for developing effective strategies to combat the virus and protect public health [5,6].

Genomic sequencing allows real-time monitoring of viral transmission dynamics by tracking sequences that aggregate together in clusters and correlating them with clinical and epidemiological data [7,8]. These data are necessary to timely inform public health about the emergence of VOCs' allowing an efficacious response [9]. Although whole-genome SARS-CoV-2 sequencing has reached an astonishing number of records, the geographic coverage is still not homogeneous, with large areas reporting only a few sequences. It is, therefore, critical to uniform sequencing coverage to monitor the molecular evolution of the virus worldwide.

The Republic of Moldova is a small Eastern European country with a population of 3.6 million [10]. The Republic of Moldova is facing a severe emigration process. Some estimate that around one million Moldovan citizens are working abroad [11], including areas of early extensive transmission of SARS-CoV-2 in Europe. On the 7th of March 2020, a 48-year-old woman who returned from Italy was the first recorded case of SARS-CoV-2 confirmed by real-time reverse-transcriptase polymerase chain reaction (RT PCR) [10,12]. In one month, the number of infected people increased to 965, with 854 cases transmitted locally and 111 imported [10,13]. Up to date (July 17, 2023), there have been 620.758 confirmed COVID-19 cases and 12.124 deaths [14].

Due to the lack of sequencing facilities in the Country, the initial strategy adopted was to partner with international institutions such as the International Centre for Genetic Engineering and Biotechnology (ICGEB) and the Charité Universitätsmedizin in Berlin, Germany [7]. Subsequently, the national diagnostic capacities of SARS-CoV-2 were expanded, and in January 2022, the National Agency for Public Health sequenced the first SARS-CoV-2 genome [15].

According to WHO recommendations, the selection criteria for sample selection for sequencing depends on the epidemiological situation, the target chosen and available resources [7].

It is indeed tragic that the COVID-19 pandemic has caused so much loss of life worldwide, including in the Republic of Moldova. The development of vaccines and antiviral drugs, as well as the use of human-neutralising antibodies (nAbs) are all essential strategies to combat the virus [16].

It is encouraging to see efforts being made to vaccinate individuals, especially those who may be at higher risk of contracting and spreading the virus, such as healthcare workers and students in communal living situations. However, it is also vital to ensure that vaccines are distributed equitably and that all individuals, regardless of occupation or social status, have access to vaccines deemed safe and effective by regulatory bodies. In March 2021, 2000 doses of the Sinopharm vaccine were donated to the Republic of Moldova, and these doses were administered exclusively to students and professors at *Nicolae Testemitanu* University [17]. Sinopharm/BBIBP-CorV is an inactivated COVID-19 vaccine that has been approved in many countries, including Moldova [17,18]. It works by triggering the immune system to produce antibodies against SARS-CoV-2. However, it is important to note that no vaccine is 100% effective, and breakthrough infections can occur even after vaccination.

While vaccination campaigns are important in preventing the spread of COVID-19, there is still a need for effective therapeutic solutions [19] to treat individuals who have already been infected with the virus, especially those who may be at higher risk of developing severe disease.

Therapeutic options for COVID-19 include antiviral drugs, such as remdesivir, and monoclonal antibodies, which are laboratory-made versions of nAbs [20]. These treatments can help to reduce the severity and duration of COVID-19 symptoms, especially when given early in the course of the disease.

Human neutralising antibodies targeting the host ACE2 receptor-binding domain (RBD) of SARS-CoV-2 have demonstrated therapeutic potential and are being tested in clinical trials [21,22]. Convalescent Plasma (CP) therapy has shown promise as a potential treatment for critically ill COVID-19 patients [23]. The FDA has recommended that CP with a virus-neutralising (VN) antibody titre of 1:160 be used for therapeutic transfusion [24]. However, the use of CP is limited by the availability of donors with high levels of VN antibodies.

Serological tests that detect nAbs to SARS-CoV-2 are crucial for monitoring the effectiveness of vaccines and for identifying individuals who may still be susceptible to the virus. Such tests can also be used to identify individuals who may have developed nAbs after being infected with SARS-CoV-2.

Neutralisation assays are considered the gold standard [18] for measuring the antiviral activity of antibodies, including nAbs, against SARS-CoV-2. However, conducting neutralisation assays with live SARS-CoV-2 virus requires the use of biosafety level 3 (BSL-3) containment facilities [25], which can be expensive and difficult to access. In addition, the handling and manipulation of live virus samples requires highly trained personnel [17] and strict safety protocols to prevent accidental exposure. To overcome this limitation, pseudotyped viruses have been developed as alternatives to infectious viruses [18,26,27]. These viruses allow for the safe and efficient testing of donor plasma or serum for their ability to inhibit virus infection. Pseudotyped viruses are engineered viruses that contain the spike protein of SARS-CoV-2 on their surface but lack the ability to replicate and cause infection. These viruses can be handled safely in biosafety level 2 (BSL-2) containment facilities, making them more accessible and easier to use in research settings [27,28].

The **aim** of this research was to study the molecular evolution of SARS-CoV-2 and the humoral immune response among Sinopharm (BBIBP-CorV) vaccine recipients and COVID-19-recovered patients in Republic of Moldova.

Research objectives:

1. Isolation of SARS-CoV-2 RNA from nasopharyngeal swabs, full genome sequencing of the isolates with high viral load, identification of genomic variants and phylogenetic analysis of SARS-CoV-2 sequences.

2. Assessment of virus neutralisation titres in samples from COVID-19 convalescent plasma donors and from serum of vaccinated people.

3. Assessment of anti-Spike RBD IgG titre in convalescent plasma donors and in serum of vaccinated people.

4. Studying the correlation between neutralising activity and anti-Spike RBD IgG antibody titres among convalescent and vaccinated individuals.

The research methodology:

The research methodology for this study involved full genome sequencing of 19 SARS-CoV-2 isolates from patients with different clinical forms and from different geographical regions of the Republic of Moldova. The isolates were selected from the Biobank of the ALFA Diagnostica laboratory based on a Confidentiality Commitment, using RT PCR reports with a threshold cycle (Ct) value lower than 30. Viral RNA was isolated by the RT PCR method in the Alfa Diagnostica laboratory. Samples with possible new mutations were prioritized to be sequenced. After RNA isolation, samples were stored at -80°C and sent to the Molecular Virology Laboratory, ICGEB, Trieste, Italy, where full genome sequencing was performed. Afterwards, metadata was created, and the results were uploaded to the GISAID international repository. Mutations of each individual

isolate were analysed, and a phylogenetic analysis was performed to understand the virus's evolutionary history in this region.

In addition to genome sequencing, it was developed a pseudotyped SARS-CoV-2 lentivirus and two neutralisation assays, one using flow cytometry and another using high content imaging, to investigate the effectiveness of the neutralising activity. An RBD ELISA test was also developed to study the level of IgG anti-Spike RBD antibodies in Sinopharm vaccinated individuals and convalescent patients. Convalescent plasma was taken from the Biobank of the National Blood Transfusion Center, and serum samples were collected from vaccinated patients. All samples were stored at -80°C, anonymized, and sent to the Molecular Virology laboratory, ICGEB, Trieste, Italy, where each sample was tested to assess the anti-SARS-CoV-2 RBD IgG antibody titre and neutralising antibody titre.

The scientific novelty and the outcome of this research

Given the ongoing COVID-19 pandemic, this study's research problem is of the uttermost importance. The scientific novelty of this project lies in the sequencing and phylogenetic analysis of the SARS-COV-2 virus from the Republic of Moldova, which can provide valuable insights into the evolution and spread of the virus in the region. Additionally, developing a pseudotyped SARS-CoV-2 lentivirus, two neutralisation assays and RBD ELISA test can help study the humoral immune response and the efficacy of vaccines against the virus.

Overall, this research can contribute to developing better tools for sera analysis and a better understanding of the immune response to SARS-COV-2 infection and vaccination, which can ultimately aid in the control and management of the COVID-19 pandemic.

Furthermore, the results obtained from this study can also help identify potential individuals who may require booster doses of the vaccine or who may have a weaker immune response to the virus. This can aid in tailoring vaccination strategies and ensuring better protection against COVID-19.

The study was **reviewed and approved** by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (Protocol No 3/24.01.22).

The research was carried out at the Department of Preventive Medicine, Discipline Microbiology and Immunology of Nicolae Testemitanu State University of Medicine and Pharmacy, Chisinau, Republic of Moldova; ALFA Diagnostica Laboratory from Republic of Moldova; Molecular Virology Laboratory, ICGEB, Trieste, Italy; AREA Science Park of Trieste, Italy.

Approval of scientific results

Research results have been presented, discussed and approved at several national and international scientific forums:

- *Workshop "Strengthening epidemiological surveillance capacity to address COVID-19 and other epidemics*", A Republic of Moldova-Italy cooperation, Online event, 21-23 September 2021;
- *1st edition of the National Conference with International participation the One Health approach in a Changing World* , Online, 4-5 November, 2021;
- *Simpozionul Național:"110 ani de la nașterea savantului George Emil Palade, tradiție și continuitate in cercetarea medicala românească".* Târgu Mureș, România, 7-8 December, 2022;
- *XV International Summer School "Biology, Biotechnology and Biomedicine"*, Odesa, Ukraine, 29 June -10 July, 2020;
- *Noaptea Cercetătorilor Europeni 2020*. https://noapteacercetatorilor.md/covid-19-ulinici-usmf, 15 November, 2020;
- *Virus Detection and Biosecurity - A Capacity-Building Course in the Framework of Article X of the BWC.* Trieste Italy, 14-16 June, 2023;
- *Tendințe actuale și provocări în medicina preventivă.* Chișinău, Republica Moldova, 8-9 June, 2023;
- *Congresul consacrat aniversării a 75-a de la fondarea USMF „Nicolae Testemiţanu*" din Republica Moldova, Chişinău, 21-23 Octomber, 2020;
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- *Emisiunea „Concret" despre vaccinurile anti-COVID și maratonul vaccinării*", TV Moldova1, 24 mai la 17:15 . https://www.facebook.com/tvmoldova1/videos/230641248427050, 24 May, 2021;
- *Emisiunea „Miezul Zilei"*, TV Moldova1, 26 septembrie, 2021. *https://www.facebook.com/teleradiomoldova/videos/395699578798680*, 26 September, 2021*;*
- *Emisiunea „Fii sănătos cu Maria Marian",* Jurnal TV, 22.11.21, ora 18:00 *https://www.facebook.com/watch/?v=183617873984484&ref=sharing*, 22 November, 2021*;*
- Interviu Sănătate Info: *http://www.sanatateinfo.md/News/Item/10934?fbclid=IwAR2GI6JGjCipGeYzKrfHWzTNEx8gm1JUigAPxqx1cjFt_aZTB8y9jHBqy64;*
- Interviu USMF: *https://usmf.md/ro/noutati/mariana-ulinici-tot-ce-realizez-este-pentru-aduce-un-aport-dezvoltarea-stiintei-din?fbclid=IwAR0qQxupojj3jr9sPKpu8cdSMBx_xEsFB14F7_iyiGW5xBF3eC8Lfhbov4s;*
- Mesager TV Moldova 1, minutul 5:40. *https://fb.watch/aSBGXOXW_6/ https://trm.md/ro/social/noi-metode-de-diagnostic-pentru-virusul-sars-cov-2;*
- Emisiunea: Pro sănătate: https://youtube.com/playlist?list=PLX… Radio Vocea Speranței Republica Moldova | Facebook, ora 18:00, 23 June, 2022.

Publications on the thesis topic

Academic portfolio comprises 15 scientific publications. This includes four peer-reviewed articles that have been accepted and published in esteemed ISI and SCOPUS. Additionally, two articles have been published in national scientific journals of category B. The entity's active engagement with the scientific community is further evidenced by nine theses that have been presented at various scientific symposia, both at the national and international level.

Key words: SARS-CoV-2 molecular evolution, immune response, mutations, variants, phylogeny, neutralising antibodies.

1. UNDERSTANDING THE BIOLOGY AND DIAGNOSIS STRATEGIES OF SARS-CoV-2

1.1. Taxonomy and Epidemiology of SARS-CoV-2

Coronaviruses are a diverse group of viruses infecting many different animals and can cause mild to severe respiratory infections in humans [29]. The current classification of coronaviruses includes 39 species from 27 subgenera, four genera, and two subfamilies that belong to the family *Coronaviridae* [30]. *Coronaviridae* belongs to the order *Nidovirales* and realm *Riboviria* and is divided into the two subfamilies *Coronavirinae (Orthocoronavirinae)* and *Torovirinae*. The subfamily *Coronavirinae* is divided into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. *Alphacoronaviruses* and *betacoronaviruses* infect mammals, while *gammacoronaviruses* and *deltacoronaviruses* primarily infect birds. SARS-CoV-2 belongs to the *Sarbecovirus* subgenus of the genus *Betacoronavirus* [31]. The genera Alpha- and Beta-coronavirus affect humans and animals. SARS-CoV (beta-coronavirus), 229E (alfa-coronavirus), HKU1 (beta-coronavirus), NL63 (alfa-coronavirus), OC43 (beta-coronavirus), and MERS-CoV (beta-coronavirus) viruses can cause infections in humans [32]. However, beta coronaviruses are the most important group, as they contain the most pathogenic viruses that can infect humans, including SARS-CoV-2, MERS-CoV, and SARS-CoV [33].

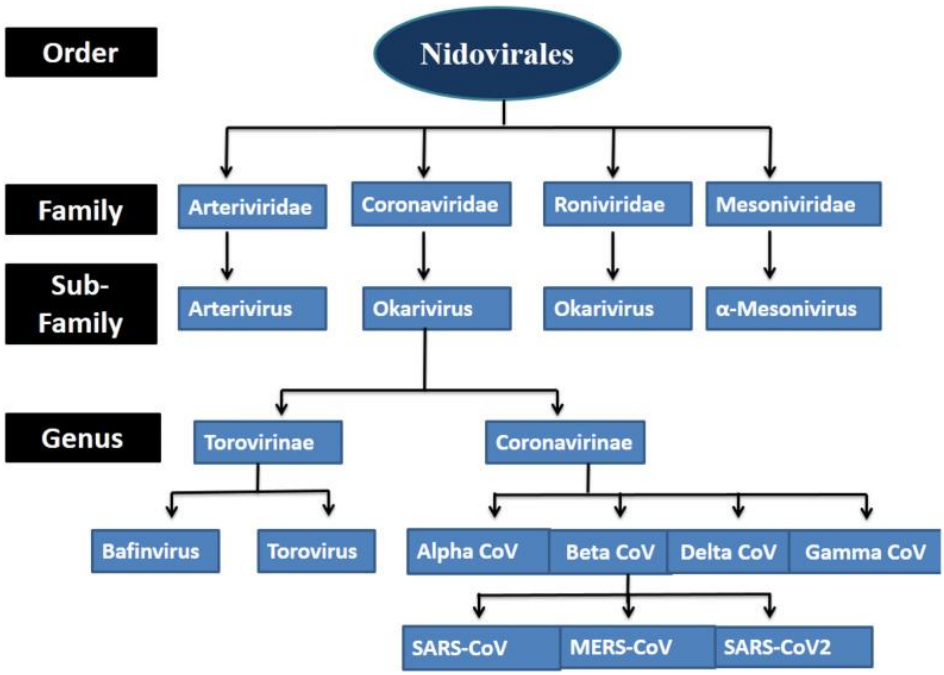

Figure 1. **Classification of coronaviruses** [34]

Viruses classified as *Nidovirales* have an unsegmented, positive-sense single-stranded RNA genome of approximately 30,000 nucleotides [35,36].

The first report attributable to the newly identified virus in Wuhan occurred on December 31, 2019. From there, the SARS-CoV-2 was seen spreading rapidly across continents (figure 2). The new virus, by then, had already begun to spread worldwide, allowing it to mutate and create numerous variants against which we were then forced to fight. Unfortunately, due to globalisation, it was impossible to contain the pandemic despite countless containment measures.

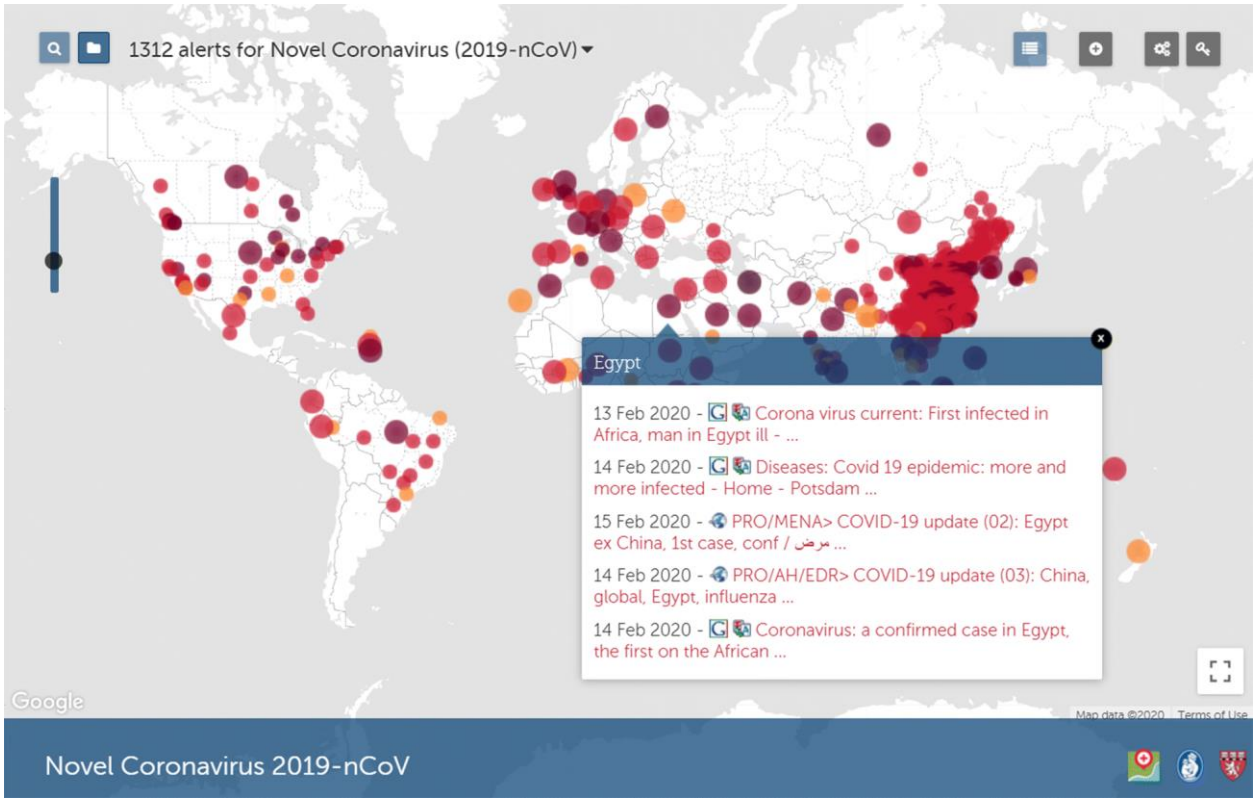

Figure 2. **Global spread of SARS-CoV-2: a visual overview** [37]

The SARS-CoV-2 virus has a spherical shape with a diameter that varies in different sources but typically ranges from 80-220 nm [38], other sources 60-140 nm [39], and 108 nm [40]. The virus's surface is covered with spike proteins that give it the characteristic "corona" or crown-like appearance. The virus uses these spikes to attach to and infect human cells. The genome of SARS-CoV-2 is a single-stranded positive-sense RNA molecule with a size of approximately 29.9 kilobases (kb) which is relatively large for an RNA virus [38,41,42].

RNA viruses generally have a high rate of mutations due to errors in the replication process, which can lead to the emergence of new variants or strains. However, SARS-CoV-2 has a unique feature in its genome - an intrinsic proofreading mechanism that helps to correct errors during replication. This mechanism is thought to be due to a non-structural protein called nsp14, which is part of the viral replication machinery. The nsp14 protein has an exonuclease activity that allows

it to detect and correct errors in the RNA during replication, resulting in a lower mutation rate compared to other RNA viruses of similar size. Initially, this proofreading mechanism was believed to result in a lower mutation rate for SARS-CoV-2, possibly only around 30 mutations per year compared to other RNA viruses [37]. However, as the virus continues to spread and infect more people, it has been found that new variants are emerging, some of which are more transmissible or virulent than the original strain. Nonetheless, the proofreading mechanism in SARS-CoV-2 is still believed to contribute to a relatively lower mutation rate than other RNA viruses, which may have important implications for developing vaccines and antiviral therapies.

As of March 27, 2023, there were over 15,280,931 complete or nearly complete genome sequences of SARS-CoV-2 publicly available, and this number continues to increase rapidly. This is an impressive feat due to the fast genome sequencing and online sharing of SARS-CoV-2 genomes by public health and research teams around the globe. These genome sequences provide valuable information about the virus's ongoing evolution (figure 3) and epidemiology (figure 4) during the pandemic. By analysing these sequences, researchers can track the emergence and spread of new variants, identify transmission patterns, and monitor changes in the virus's genetic makeup over time. This information is critical for developing and updating vaccines, treatments, and diagnostic tests for SARS-CoV-2 and for guiding public health interventions aimed at controlling its spread. By continuing to sequence and share SARS-CoV-2 genomes, researchers can better understand the virus and its impact on global health.

According to Mascola (2021), there are three definitions of the terms related to the genetic evolution of viruses such as SARS-CoV-2 "mutation," "variant," "strain," and "lineage" [43].

A mutation refers to a change in the virus's genetic sequence, which can lead to a change in the amino acid sequence of viral proteins. Non-synonymous mutations specifically refer to changes in the amino acid sequence that result in a change in the amino acid sequence of a protein. These mutations can occur spontaneously during the replication of the virus, or they can be induced by external factors such as host immune responses or antiviral treatments. **A variant** refers to a group of mutations that occur together in a single viral genome, and a confirmed variant with unique properties is referred to as a strain. Variants can arise due to natural selection, genetic drift, or recombination events between different viral strains. Finally, the term **lineage** is used in the context of phylogenetic analysis to describe the evolutionary relationships between different viral variants. Lineages are identified based on the pattern of mutations that they share and are typically represented as branches on a phylogenetic tree [43].

To date, several variants of SARS-CoV-2 have been emerged. These can be designated as Variants of Concern (VOCs), Variants of Interest (VOIs), and Variants under Monitoring (VUMs). VUMs are those variants that are rare or circulating at deficient levels. VOIs are variants with

genetic markers associated with changes in receptor binding, reduced neutralisation by antibodies generated against previous infections and/or vaccines, reduced efficacy of treatments, potential diagnostic impact or expected increase in transmissibility or severity of the disease. On the other hand, VOCs are variants for which there is an increase in transmissibility, virulence, or ability to evade immune responses. The most well-known VOCs are Alpha, Beta, Gamma, and Delta, which have all been shown to have mutations in the spike protein that increase their ability to infect cells or evade antibodies. Currently, only one VOC is circulating. The variant was named Omicron and comprises B.1.1.529, BA.1, BA.2, BA.3, BA.4, BA.5, and descendent lineages. Previous circulating VOCs are Alpha, Beta, Gamma, and Delta [44]. Evolutions in the trend of infections, such as discoveries or new therapies, can lead to changes in the classification of variants over time; as a result, the latest VOCs are replaced by other co-circulating SARS-CoV-2 variants. Delta reached almost 90% of all viral sequences submitted on GISAID by October 2021. Omicron is currently the dominant variant circulating globally, accounting for >98% of the viral sequences shared on GISAID after February 2022 [44].

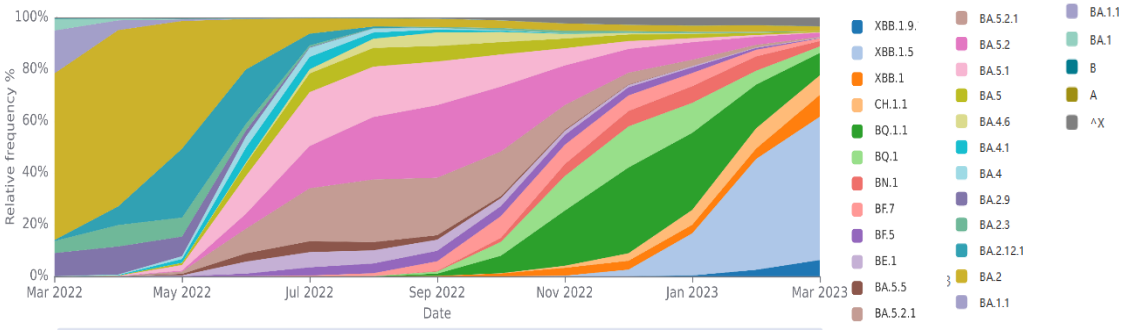

Figure 3. **SARS-CoV-2 lineage relative frequencies over time** (retrieved from GISAID, 27 March 2023)

The Delta variant (Variant VUI-21APR-01, also known as B.1.617) was first detected in India and reached almost 90% of all viral sequences submitted on GISAID by October 2021. The B.1.617 variant is characterised by a series of mutations in Spike glycoprotein, including E484Q, L452R, and P681R, and has shown transmissibility ranging from 40 to 60% higher than the Alpha variant; it has also proved more infectious in unvaccinated or partially vaccinated subjects than the other variants [45]. The Omicron variant (Variant B.1.1.529), instead, was first detected in South Africa in November 2021 and became the dominant variant circulating globally after February 2022 (figure 4). Variant B.1.1.529 has more S-gene mutations than the original virus or other variants in circulation. Because of these Spike mutations (more than 30), there is concern that they may significantly change the antigenic properties of the virus [46,47]. Although to date, the possibility of changes in transmissibility, the severity of infection, or potential evasion of the

immune response is still an argument of many studies [48]. The molecular evolution of SARS-CoV-2 is of great interest to scientists as it can provide insight into the virus's origins, transmission, and virulence.

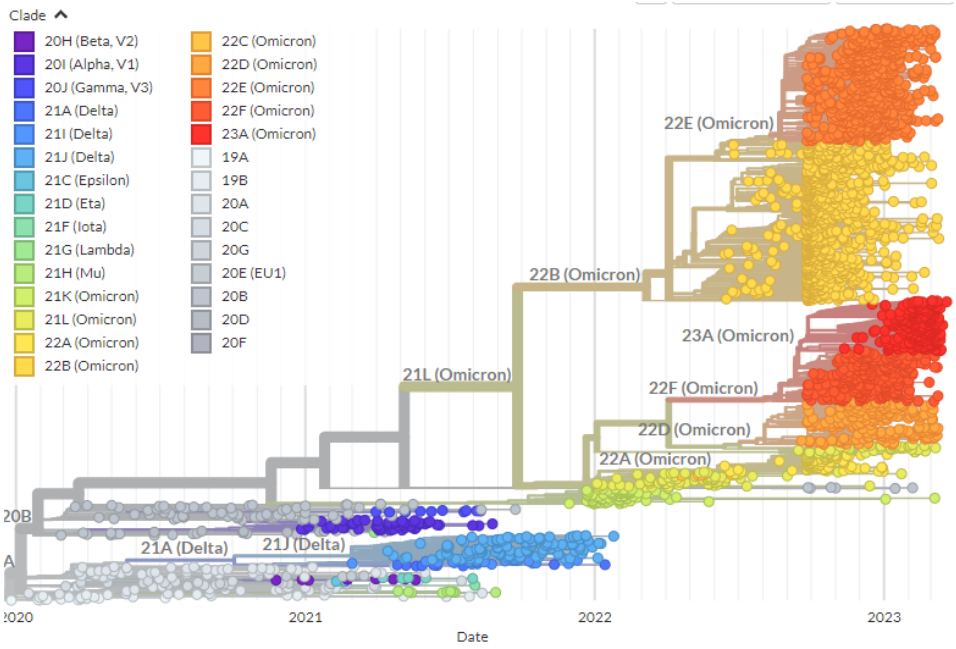

Figure 4. **Genomic epidemiology of SARS-CoV-2 with subsampling focused globally** (27 March 2023, available at nextstrain.org/sars-cov-2.)

1.2. SARS-CoV-2 genomic organisation

Like other coronaviruses, SARS-CoV-2 is an enveloped, single-stranded positive-sense RNA virus with a non-segmented genome of approximately 30 kb. The genome is flanked by a 5'-cap and a 3'-poly-A tail, permitting it to act as functional mRNA able to undergo translation during protein synthesis. It contains 5'-UTR and 3'-UTR (untranslated regions), forming multiple secondary loop structures involved in the replication. The viral genome encodes for 16 nonstructural proteins (NSPs) required for virus replication and pathogenesis and four structural proteins [49] that play various roles in the virus's life cycle and pathogenicity. The four structural proteins of SARS-CoV-2 are the membrane (M) protein, spike (S) glycoprotein, envelope (E) protein, and nucleocapsid (N) protein. The M protein is responsible for virus assembly and morphogenesis, while the S glycoprotein is the best-known protein and provides three major functions: recognition, attachment, and fusion with host cell receptors. The E protein is a small protein that plays a role in mediating virus engulfment and release [39] by forming a channel through the endoplasmic reticulum membrane and the Golgi intermediate compartment [40]. The N protein, which surrounds the genome, binds to genomic RNA in the virion and packages it into the ribonucleoprotein (RNP) complex. The N protein has three regions: the N-arm, central region,

and C-tail, as well as two other structural domains, the N-terminal domain (NTD) and the C-terminal domain (CTD). The NTD is responsible for RNA binding, while the CTD is involved in dimerization. The SARS-CoV-2 genome is larger than most RNA viruses, and it is thought that the RNPs allow for high steric flexibility, which allows for efficient viral genome packaging into virions [40].

The genomic RNA of SARS-CoV-2 contains 14 ORFs, particularly ORF1a and ORF1b, constituting two-thirds of the entire genome and translating into the polyproteins pp1a and pp1ab, respectively. The viral protease then processes these to produce the 16 non-structural proteins (NSPs), each performing a distinct function in the virus's life cycle. Some of the functions of these NSPs include ribosomal RNA processing and replication (NSP1), host cell survival signalling (NSP2), translated protein cleavage with host DNA-ribosylation disruption (NSP3), and transmembrane domain formation (NSP4) [40].The major protease of SARS-CoV-2, responsible for cleaving and processing viral polyproteins, is NSP5. The NSPs 7, 8, and 12 work together to form a holo-RdRp complex, with Nsp12 acting as the RNA-dependent RNA polymerase, the key enzyme required for viral genome replication and transcription [40]. Other important NSPs include NSP13, which is involved in replication and transcription and participates in ATP binding, and NSP14, which has a proofreading exoribonuclease activity that helps to maintain the integrity of the viral genome. NSP15 functions as an Mn2+-dependent endoribonuclease, while NSP16 acts as a 2-O-ribose methyltransferase, playing a crucial role in the formation of mRNA caps [41].Some of these NSPs form the replication-transcription complex (RTC), which is required for the replication and transcription of the viral genome. The remaining third of the genome encodes for the four major structural proteins: Envelope (E), Membrane (M), Nucleocapsid (N), and Spike (S) [50]. The nucleoprotein (N) surrounds the RNA genome to produce a tubular spiral structure. The viral envelope (E) covers the helical nucleocapsid. Two or three structural proteins are associated with the viral envelope. The matrix protein (M) is embedded in the viral envelope, while the Spike structural protein (S) is anchored in the viral envelope and is the target of the neutralising antibody. Many beta-coronaviruses also contain hemagglutinin esterase [33,51].

- ***Glycoprotein S (spike)***

Structure of glycoprotein S

The SARS-CoV-2 Spike protein is one of the most studied proteins since it is the infectious component of the virus. The infectivity of coronaviruses devoid of Spikes seems low [52].Consequently, this protein is crucial for viral entrance since it binds to Angiotensin Converting Enzyme [53]. It contains the receptor binding domain (RBD) responsible for interacting with the host cell membrane with epitopes recognized by B and T lymphocytes, which

will subsequently induce antibody production [38]. The S-glycoprotein is a prominent type 1 viral fusion protein that is approximately 24 nm long and trimeric, with a total molecular weight of around 600 kDa. It comprises two subunits, S1 and S2, that are formed through proteolytic cleavage of a precursor protein. The surface of the S-glycoprotein is highly glycosylated, with 22 N-linked glycosylation sites per monomer [40], which is thought to play a critical role in proper protein folding and neutralisation by antibodies [41]. The S-glycoprotein is highly flexible and appears as a flexible head placed on a stalk that can be tilted by up to 90 degrees to its own membrane. This flexibility, combined with the glycan envelope, allows the virus to quickly scan the host cell surface and conform to its receptor, ACE2, facilitating viral entry into host cells [40].

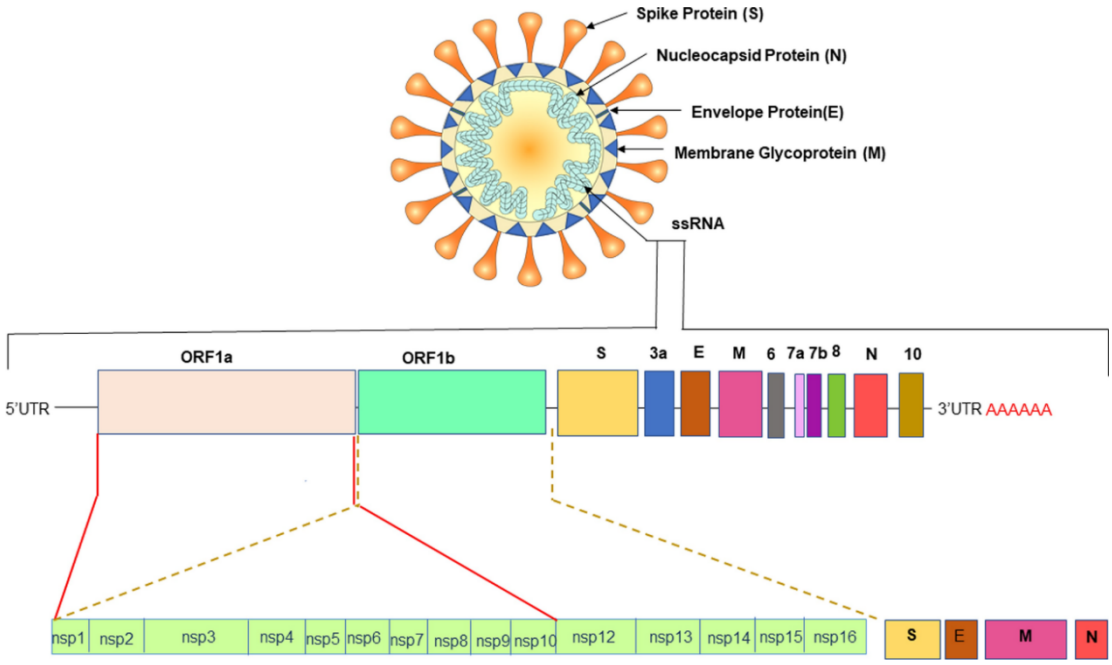

Figure 5. **Visualization of the genomic architecture of SARS-CoV-2** [51]

The surface subunit S1 is organised into four domains: an N-terminal domain (NTD), a C-terminal domain (CTD, also known as the receptor-binding domain, RBD), and two subdomains (SD1 and SD2). The transmembrane S2 subunit is composed of 588 amino acids (residues 686-1273) [54]. It contains an N-terminal hydrophobic fusion peptide (FP), two heptad repeats (HR1 and HR2), a transmembrane domain (TM), and a cytoplasmic tail (CT), arranged as FP-HR1-HR2-TM-CT [55]. Spike is involved in the binding and fusion of the virus to the target cell. Because of its primary and fundamental function, this glycoprotein is being studied as a target region for the action of vaccines and therapies [56].

Understanding the structure and function of the SARS-CoV-2 S protein is critical for the development of effective vaccines and therapies to combat COVID-19 [55].A schematic

representation of the domain arrangement of the SARS-CoV-2 S protein precursor is shown in figure 6, which illustrates the different regions and domains of the spike protein. These regions include the signal peptide (SS), the N-terminal domain (NTD), the receptor-binding domain (RBD), the receptor-binding motif (RBM), subdomains 1 and 2 (SD1/2), the fusion peptide (FP), heptad repeat 1 (HR1), the central helix (CH), the connector domain (CD), heptad repeat 2 (HR2), the transmembrane domain (TM), and the cytoplasmic tail (CT). Arrows denote the protease cleavage sites.

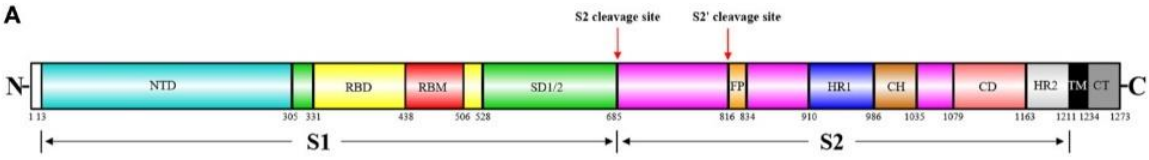

Figure 6. **Structural features of the SARS-CoV-2 Spike Protein** [55]

One of the unique features of SARS-CoV-2 compared to other coronaviruses is that the S-glycoprotein is cleaved into subunits by a furin-like protein convertase during biosynthesis in virus-producing cells. In contrast, cleavage occurs in other coronaviruses only when the virus reaches the next cell [58]. The S-glycoprotein plays a crucial role in the entry of the viral genome into the cytoplasm of the infected cell, and the cleavage of an additional site on the S2 subunit facilitates this entry. Once this additional site is cleaved by the host cell protease TMPRSS2, the fusion protein is released, and pores are formed through which fusion will take place, allowing the viral genome to access the cytoplasm. Nevertheless, this will only happen when the pore expands and the viral and cellular membranes merge seamlessly [58]. Host proteases must cleave glycoprotein S at the S2 cleavage site for all coronaviruses to activate proteins, which is essential for membrane fusion [41].

The S-glycoprotein of SARS-CoV-2, which is fundamental to the virus, assumes two vital conformations: the pre-fusion and post-fusion states (as depicted in figure 7). These conformational states are crucial for the virus's ability to enter host cells. In the pre-fusion conformation, the S1 subunit folds into four domains that wrap around the S2 subunit, forming a central helical bundle with heptad repeats (HR1). Three RBDs at the tip of the S protein determine the two conformations - the "up" conformation that is accessible for receptor binding and the "down" conformation that is inaccessible and cannot recognize ACE2 on the host cells. After fusion, the S1 and S2 subunits uncouple, and the S-glycoprotein transitions to the post-fusion conformation [57]. In the "closed" state, the three recognition motifs do not leave the interface formed by three protomers of the spike protein [41]. In the post-fusion conformation, HR1

undergoes a transition allowing the introduction of the fusion peptide (FP) [58], and a long central coil with three coiled chains is formed [59]. HR2 folding places FP and the transmembrane domain at the same end, allowing the interacting membranes to fold towards each other and merge, allowing the viral genome to enter the host cell's cytoplasm. The post-fusion conformation is rigid, stable, and highly enriched in N-linked glycans, which participate in immune evasion. If all glycans are occupied by glucose, most surfaces will be protected from antibody action, contributing to the virus's ability to evade the immune system [58].

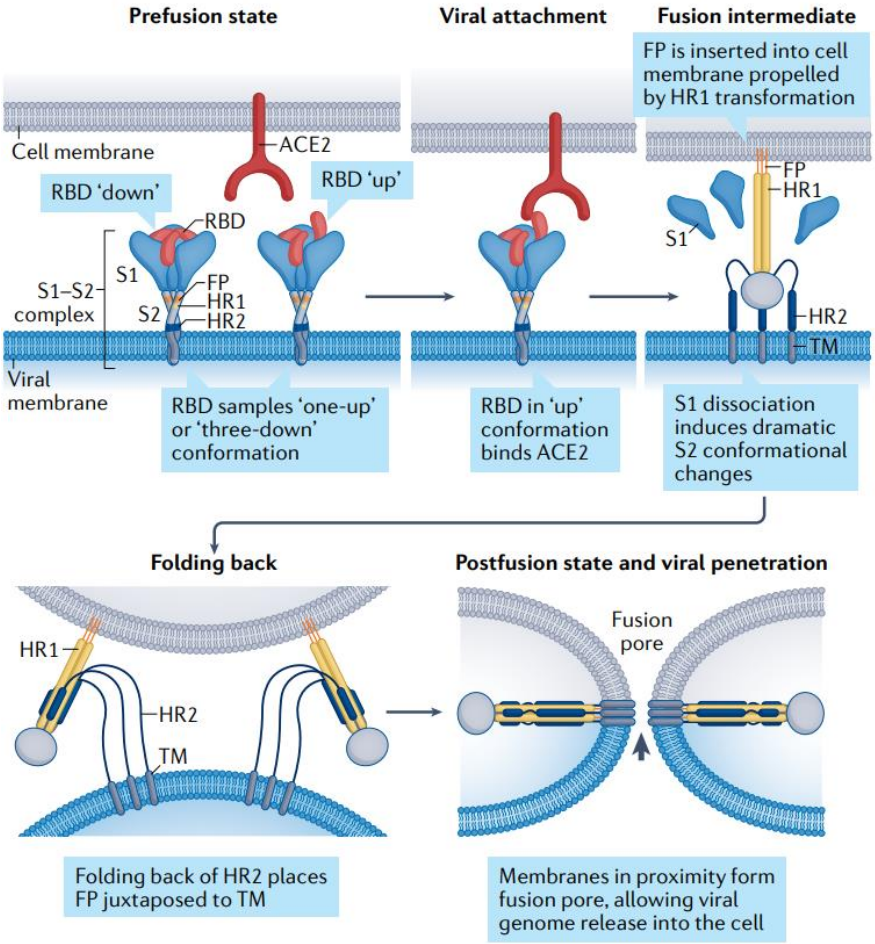

Figure 7. **The mechanism of membrane fusion by SARS-CoV-2 protein S** [57]

The 6-HB structure, which is formed by the interaction between HR1 and HR2, is critical in mediating membrane fusion during viral entry. Studies have shown that differences in the 6-HB structure between SARS-CoV and SARS-CoV-2 are one of the factors that contribute to the increased infectivity of the latter [41].For example, SARS-CoV-2 has improved interaction between HR1 and HR2 due to forming a stronger hydrogen bond between HR1 and HR2 serine, while SARS-CoV has a lysine binding to glutamic acid via a salt bridge. Another difference is that in SARS-CoV, HR1 glutamine does not bind to HR2 at all, whereas in SARS-CoV-2, a salt bond

is formed between lysine HR1 and asparagine HR2. These structural differences may contribute to the increased infectivity of SARS-CoV-2 compared to SARS-CoV [41].

Receptor Binding Domain of SARS-CoV-2 Spike Protein

The RBD (amino acids 319-531 of protein S) is the most important component of the spike glycoprotein and the main target of neutralising antibodies, as it accounts for more than 90% of all neutralising activity [57]. The RBD has two subdomains - a core structure and an extended loop that wraps around the edge of this core structure, forming the receptor-binding motif (RBM). The RBM makes all the contacts with the ACE2 receptor, and its accessibility determines the conformation of the S protein and its ability to bind to the receptor. In the bottom conformation, a single RBD packs onto the helical bundle of S2, while resting on CTD1 from the same protomer and NTD from a neighboring one. This conformation partially occludes the RBM, leading to receptor inactivation. Upon transition to the top conformation, the RBM, CTD1, and NTD become completely exposed, allowing the S protein to bind to the ACE2 receptor and mediate viral entry into host cells.

The RBD is made up of five anti parallel B-sheet filaments (B1...B5) surrounded by short helices and the receptor binding motif (RBM). The RBM forms a loop that protrudes from the primary core and creates a cradle structure essential for receptor binding. There are two conformations of the RBD, which depend on its mobility. The down conformation is for hidden RBD, while the up conformation is for exposed RBD. The Spike can only interact with the receptor in the up conformation. The ACE2 receptor has an N-terminal helix that interacts with the RBM, and this interaction is stabilized by 14 hydrogen bonds and a salt bridge [41].

The RBD is a critical target for neutralising antibodies, and most antibodies recognize the top region of the RBM, including casirivimab (REGN10933) and C144. Other RBD sites, including a site in the bottom conformation, are recognized by imdevimab (REGN10987), S309, and the supersite in the top conformation RBD is recognized by CR3022. These epitopes constitute the majority of targets for SARS-CoV-2 neutralising antibodies. Regarding the S2 subunit, HR1 undergoes the most dramatic refolding upon transition from the pre-fusion to the post-fusion conformation. This refolding introduces the fusion peptide into the target cell membrane, leading to the formation of a pore and the entry of the viral genome into the cytoplasm [57]. Understanding the mechanisms of S protein conformational changes is essential for developing treatments and vaccines targeting these critical virus components and understanding the mechanisms of viral entry and pathogenesis.

Mutations in the RBD may alter receptor binding affinities and affect the host range of the virus. By analyzing the mutational dynamics of the RBD of SARS-CoV-2 against the RBD of closely related CoVs, researchers confirmed that six amino acids had been changed, possibly altering the host range. However, sequence and structural comparisons of the RBD and ACE2 suggest that SARS-CoV-2 RBD is well suited for binding to ACE2 from humans and other species with high receptor homology [59].

D614G Variant

The D614G mutation is a single nucleotide polymorphism (SNP) that results in the substitution of an aspartic acid (D) with a glycine (G) at position 614 of the spike protein. This mutation was first identified in Europe in early 2020 and quickly spread to become the dominant form of the virus in many countries around the world [60]. Studies have shown that the D614G substitution in the SARS-CoV-2 spike protein may affect the conformational stability of the spike protein and enhance its binding to the ACE2 receptor, which is the receptor for viral entry into host cells. Specifically, the D614G mutation is thought to disrupt a hydrogen bond interaction between the D614 residue and a threonine residue (T859) in a neighbouring protomer of the spike protein trimer. This disruption may allosterically shift the RBD to an "up" conformation, increasing the accessibility of the RBD for binding to the ACE2 receptor, leading to enhanced virion infectivity [61,62] than the original strain of SARS-CoV-2. One study found that the D614G variant was associated with higher patient viral loads, suggesting that the variant may be more infectious [60]. Another study found that the D614G variant was associated with higher replication rates in human respiratory cells, indicating that the variant may be more efficient at infecting and replicating in host cells [63]. Although the D614G mutation in the SARS-CoV-2 spike protein has been shown to increase infectivity, several studies have found no significant difference in disease severity between patients infected with the D614G variant and those infected with the original strain of SARS-CoV-2 [64]. However, further research is needed to fully understand the potential implications of the D614G mutation on the clinical course of COVID-19.

Omicron variant

The Omicron variant of SARS-CoV-2 has raised concerns due to its high number of mutations, which could affect its transmissibility and immune evasion capabilities. In addition to the four key mutations (K417N, Q493R, N501Y, and Y505H) in the RBD region (residues 319-541), Omicron also carries mutations in other regions of the Spike protein, including the N-terminal domain (NTD), which are involved in the enhancement of the binding ability of the virus to hACE2 and may also contribute to increased transmissibility (figure 8) [47]. These Spike mutations increase the Omicron variant's spread, decrease the binding capacity of the neutralising

antibody, and increase the Expression of viral RNA. The result of these modifications on Spike confers substantial advantages in the competition of Omicron with other variants. These additional mutations and deletions in other genomic regions may also have an impact on the virus's biology, but their functional significance is not yet fully understood. Some of these mutations have been previously identified in other variants, while others are unique to the Omicron variant.

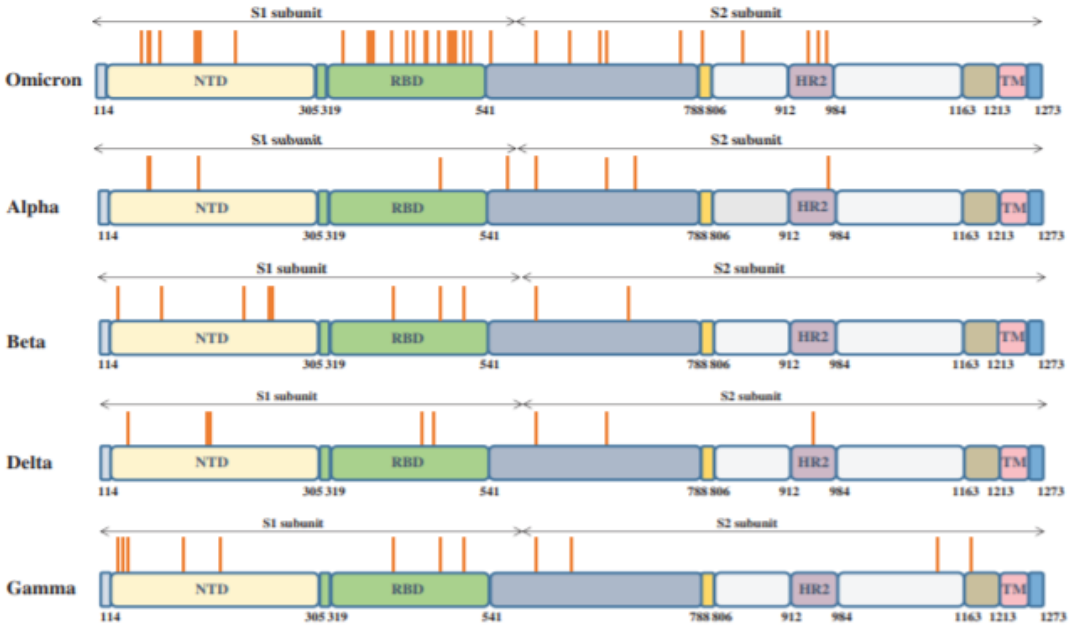

Figure 8. **Amino acid mutations on the S glycoprotein in different SARS-CoV-2 variants** [65]

Therefore, the Omicron variant has the potential to trigger a new global epidemic peak. Variant B.1.1.529 (Omicron) is characterised by 30 amino acid changes, three small deletions, and a small insertion in the Spike protein compared with the original virus (A67V, Δ69-70, T95I, G142D, Δ143-145, Δ211, L212I, ins214EPE, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493K, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F) [47]. The variant also involves several modifications and deletions in other genomic regions (NSP3 - K38R, V1069I, Δ1265, L1266I, A1892T; NSP4 - T492I; NSP5 - P132H; NSP6 - Δ105-107, A189V; NSP12 - P323L; NSP14 - I42V; E - T9I; M - D3G, Q19E, A63T; N - P13L, Δ31-33, R203K, G204R) [66].

It is important to note that not all mutations in the Omicron variant will significantly impact the virus's behaviour and that further studies are needed to understand these mutations' implications fully.

1.3. Adaptive immunity in SARS-CoV-2 infection

Adaptive immunity is the body's immune response to a viral infection that develops over time. This type of immunity involves activating and proliferating specific immune cells, such as B cells and T cells, that can specifically recognize and target the virus. B cells produce antibodies that bind to and neutralize the virus, while T cells attack and kill infected cells. The magnitude of T and B cell responses is determined by factors such as the virus's pathogenicity, the extent of inflammation, and the kinetics of viral replication. Once the infection is cleared, a population of memory B and T cells remain in the body, providing long-lasting protection against future infections with the same virus. This process is the basis for developing vaccines, which stimulate the immune system to produce memory cells without causing disease [67].

Humoral immune response

The humoral immune response is a significant part of the adaptive immune response and produces antibodies that can specifically bind to the virus and neutralize it, preventing it from infecting new cells. Serum neutralisation stops the virus from spreading to uninfected cells and allows other defence mechanisms to clear the infection. Follicular helper T cells support B cell maturation and antibody production. CD4+ cells differentiate into follicular helper T cells, which differentiate into antibody-producing cells, which block the virus from entering the host cell and cause lysis of the infected cell and the development of memory B cells. Once the virus has been neutralized, other immune mechanisms can clear the infection. Innate and adaptive immunity coordinate and work together for one ultimate goal, namely, to decrease viraemia and reduce inflammation, which usually happens over a week. This coordination between innate and adaptive immunity is crucial for resolving viral infections and generating long-term immunity [67].

Naïve T and B cells demonstrate a broad scale of viral recognition due to the broad spectrum of T cell receptors (TCR) and B cell receptors (BCR). Clones with high-affinity TCR are chosen during infection because they have a high capacity to bind viral peptides. In contrast, the affinity of BCRs continues to increase during infection.

The primary function of serum neutralisation is to prevent the virus from spreading into healthy cells, thus allowing the infection to be cleared. Immunoglobulin G (IgG), immunoglobulin M (IgM) and secretory immunoglobulin A (IgA) are the three types of antibodies that will be produced following viral infection. CD4+ cells promote germinal centre B-cell class-switching of the immunoglobulin class from early IgM production to high-affinity IgG and IgA (figure 9). IgA is primarily found on mucosal surfaces, such as in the respiratory and gastrointestinal tracts, where it acts as the first line of defence against viruses by preventing them from binding to and entering host cells. IgG, on the other hand, is responsible for directly neutralising the virus in circulation

by binding to its surface proteins and preventing it from infecting host cells. After binding to the virus, IgG can also activate immune cells, such as mononuclear cells and leukocytes, to phagocytose the virus. The complement cascade can also be activated by antibody binding, which can help to strengthen early responses to infection, mainly when antibody levels are low or when the virus has evolved to evade antibody recognition [67]. Several studies have shown that the magnitude of the initial antibody response, as measured by IgM and IgG antibody titres, correlates with the severity of the infection [68].

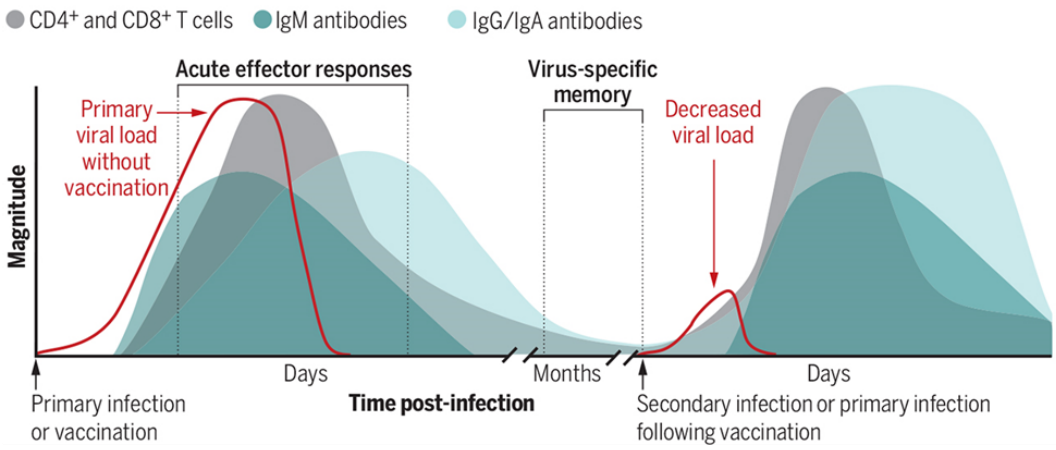

Figure 9. **Antiviral adaptive humoral immune response** [67]

The transition from innate to acquired immunity is critical in the SARS-CoV-2 immune response, resulting in either a protective or an aggravated inflammatory immune response. Ideally, all actors in the adaptive immune response should be ready for infection management quickly after the commencement of the non-specific immune response. Although the intrinsic role of innate immunity in immunopathogenesis has yet to be fully explained, there is some evidence of the role of pathogenic and specific immunity [39]. It is vital to highlight that epitope dissemination is more commonly related to autoimmune illnesses than viral infections. The immune response incorrectly targets self-antigens in autoimmune disorders, resulting in tissue damage. As tissue is damaged, more self-antigens are released, causing the immune response to spread to new epitopes, a process known as epitope spreading. In the case of SARS-CoV-2 infection, however, tissue death results from viral replication and the associated immune response rather than an autoimmune process.

T-cells, CD4 cells that assist antibody-producing B cells, are essential for immune defence. Antibodies targeting the RBD protein S are known to prevent virus attachment to vulnerable cells. IgM and IgA antibodies are discovered as early as the first week, while IgG antibodies are detected two weeks later. Cross-reactivity has also been seen between SARS-CoV and SARS-CoV-2, which share 90% of the spike protein's amino acids. It is also possible that those who had an earlier

immune response had cross-reactive antibodies to non-SARS-CoV coronaviruses. As a result, while neutralising antibodies does not correlate with disease severity, circulating follicular T cells, which are progenitors to neutralising antibodies, do [39].

CD4 T cells have substantially stronger antiviral activity than CD8 T cells and regulate disease severity better. CD4 T cells have multiple targets with sequentially low expressivity, with the highest being S, M, and N antigens [39]. Non-specific proteins, such as Nsp3, Nsp4, and ORF8, also have high specificity [69]. During the early stages of the immune response, CD4+ T cells do differentiate into different subsets, including Th1 cells, which play an important role in the immune response against viral infections. Follicular helper T cells (Tfh) are also important to produce high-affinity antibodies by B cells [70]. CD8 T cells are activated and expanded from pre-existing memory cells upon encountering viral antigens presented on infected cells. This activation and expansion process typically takes several days after initial exposure to the virus. Once activated, CD8 T cells can produce cytotoxic molecules such as IFN-γ, perforin, and granzyme B to kill infected cells [71].

Once the virus has been eradicated, memory B cells, memory T cells, and long-lived antibodies will provide protection against future infections. There are central memory cells that can regenerate themselves. Other human coronaviruses have been shown to generate SARS-CoV-2 reactive memory T cells. Similarly, antibodies reactive to SARS-CoV-2 were found in people who couldn't tolerate COVID-19. An increased innate response with hyperinflammation will result in T lymphocyte activation, depletion, immunosuppression, and reduced viral clearance [67]. Lymphocytopenia is present at the periphery, but the remaining lymphocytes are highly active, and severity worsens in connection with low TFH [72].

The humoral immune response to SARS-CoV-2 infection is complex and can vary widely among individuals. Some infected individuals may remain asymptomatic, while others may develop severe symptoms and require hospitalization. Studies have reported that patients with severe COVID-19 tend to have higher levels of IgM and IgG antibodies compared to those with mild or asymptomatic infections [73–76]. According to Lucas et al. (2020) deceased patients mounted a robust but delayed humoral response, including anti-spike IgG, anti-receptor-binding domain (RBD) IgG, and neutralising antibody (NAb). In addition, they asserted that the production of NAb before to 14 days following the onset of illness is a critical recovery factor [77]. According to a study looking for early disease indicators, spike-specific humoral responses are concentrated in convalescent patients, whereas functional antibody responses to the nucleocapsid are stronger in deceased individuals [78].

Recent studies have also shown that the humoral immune response to SARS-CoV-2 infection is long-lasting. A study published in Nature Communications reported that the levels of

anti-SARS-CoV-2 antibodies remained stable for at least six months after infection [79]. Another study published in Frontiers in Medicine found that the levels of antibodies remained high for more than one-year post-infection [80].

Antibodies to SARS-CoV-2 can target various proteins encoded by the virus, including both structural and non-structural proteins. However, the two structural proteins most commonly used as target antigens for serological assays are the nucleoprotein (NP) and the spike protein. The nucleoprotein is abundant and found inside the virus or infected cells, but due to its biological function and the fact that it is shielded from antibodies by viral or cellular membranes, it is unlikely that NP antibodies can directly neutralize SARS-CoV-2. On the other hand, the spike protein is a large trimeric glycoprotein that contains the receptor binding domain (RBD), which the virus uses to dock to its cellular receptor ACE2 and for fusion of viral and cellular membranes. Antibodies to the spike protein, particularly those that bind to the RBD, have been shown to be effective at neutralising th virus and preventing infection. P2B-2F6 has strong competitiveness and binding affinity to both upward and downward conformations of RBD, making it a promising candidate for providing prophylactic and therapeutic protection against COVID-19 [81]. Therefore, the spike protein is a more relevant target for evaluating the immune response to SARS-CoV-2 [21].

Neutralisation is one of the most significant roles of antibodies, as it induces sterile immunity against viral infection [82]. However, there is evidence to suggest that the humoral immune response to SARS-CoV-2 infection may be impacted by the emergence of new variants of the virus. Studies have shown that some variants, such as the Beta, Gamma and Omicron variants, can partially escape the neutralising antibodies produced in response to the original strain of SARS-CoV-2 [83].

Antibodies are the first and most likely target for vaccine development because they can wipe out immunity. In severe COVID-19 patients, the T-cell response is always weak, and the number of T cells is drastically reduced. However, the humoral responses to SARS-CoV-2 are different, and their correlations with disease severity are not always the same.

Several studies of SARS-CoV-2 showed that IgM and IgG antibodies appear simultaneously, around ten days from the infection (figure 10) [84].

The studies carried out since the beginning of pandemic reveal that all age groups seem susceptible to SARS-CoV-2 infection. Specifically, in the age of 50 years, most cases are asymptomatic or mild disease, but after the age of 60, the condition seems to worsen, especially in patients with other ongoing pathologies [85]. There is also evidence to suggest that the humoral immune response to SARS-CoV-2 infection may be affected by age, gender, and underlying health conditions. A study published in Nature Aging found that older individuals had lower levels of anti-SARS-CoV-2 antibodies than younger individuals [86]. Qi et. al. (2021) reported in a study

that men have a higher risk of severe COVID-19 illness and death than women. One possible explanation for this is that men have a more robust innate immune response, which can lead to an overreaction of the immune system, known as a cytokine storm. Cytokine storms can cause severe inflammation and damage to organs, which can be fatal. In contrast, women may have a stronger adaptive immune response to COVID-19. Women tend to produce more antibodies in response to the virus than men. This may provide them with better protection against future infections [87].

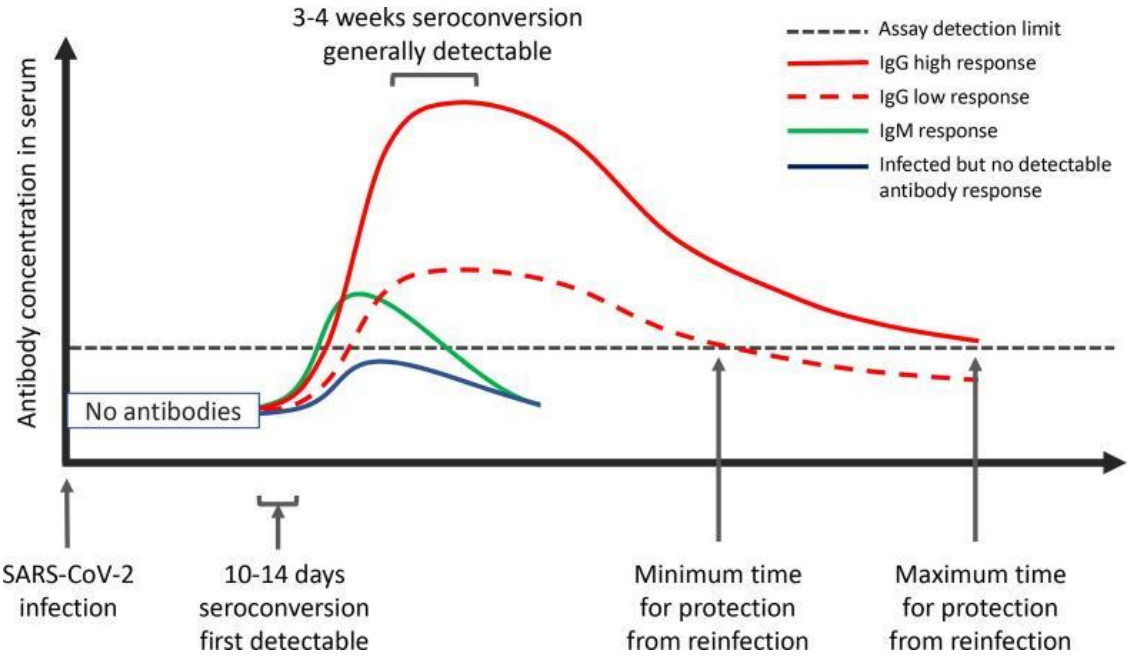

Figure 10. **A visual overview of the immune response to SARS-CoV-2 infection** [84]

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Antiviral cellular immune response

It is certain that the role of the cellular immune response is clearly superior to the humoral one. Cellular immunity comes with two key moments: cytotoxic T lymphocytes and antibody-dependent cellular cytotoxicity.

Additionally, cytotoxic T cells can also eliminate virus-infected cells through the Fas-FasL pathway. This pathway involves the binding of FasL on the surface of the T cell with Fas on the surface of the infected target cell, leading to activation of caspase and initiation of apoptosis. This mechanism is particularly important in the elimination of virus-infected cells that do not express HLA-I on their surface, such as in cases of viral immune evasion. In addition, cytotoxic T cells can also secrete cytokines such as interferon-gamma (IFN-γ) that have antiviral effects and activate other components of the immune system. Overall, the cellular immune response plays a critical role in controlling viral infections and preventing viral persistence [88].

On the other hand, antibody-dependent cellular phagocytosis (ADCP) is a process where phagocytic cells such as macrophages and neutrophils recognize and engulf antibody-coated target cells or particles. The Fc region of the antibodies bound to the target cell is recognized by Fc receptors on the surface of the phagocytic cell, triggering phagocytosis and destruction of the target. ADCP is also an important mechanism for eliminating pathogens and infected cells [88].

1.4. Strategies for diagnosis of SARS-COV-2

Early diagnosis is essential to identify the infection and prevent the spread of the virus.

To gain a better understanding of SARS-CoV-2 evolution and the potential impact of within-host immune selection on viral diversification, it is crucial to examine viral sequences obtained at different stages of infection and from different tissues. This would provide valuable information on how the virus evolves and adapts to the host immune response. It is also important to consider the role of convergent evolution, where multiple independent lineages develop the same or similar mutations due to similar selective pressures. This phenomenon has been observed in SARS-CoV-2, with multiple variants emerging across the globe, sharing some key mutations in the spike protein, such as N501Y, which is found in the B.1.1.7 (Alpha), B.1.351 (Beta), and P.1 (Gamma) variants. These mutations enhance viral transmissibility and, in some cases, can affect neutralisation by antibodies. Longitudinal studies that collect viral samples from patients over the course of infection would help reveal the dynamics of viral evolution within hosts. Moreover, sequencing viruses from diverse geographical regions can provide a more comprehensive understanding of how SARS-CoV-2 evolves in response to population-specific factors, such as local immunity and environmental conditions.

Studying viral evolution in immunocompromised individuals may also offer insights into the selection pressures experienced by the virus, as these patients often exhibit prolonged infections, allowing the virus to replicate and accumulate mutations. In some cases, these mutations could lead to the emergence of new variants with increased transmissibility or immune evasion capabilities.

Understanding the factors that drive SARS-CoV-2 evolution is crucial for informing public health strategies, vaccine development, and therapeutic interventions. By identifying key mutations and their effects on viral fitness, researchers can better predict the emergence of new variants and develop strategies to mitigate their impact on public health. From May to November 2020, four significant variants of SARS-CoV-2, known as VOCs, emerged in quick succession - Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1.), and Delta (B.1.617.2). However, the subsequent major VOC, Omicron (B.1.1.529), was not identified until November 2021, and there have been no further VOCs discovered since then. As a result, much effort has gone into understanding Omicron sublineage evolution and its impact on vaccine-induced and convalescent immunity escape, as well as the acute and long-term pathologic sequelae of COVID-19 [89].

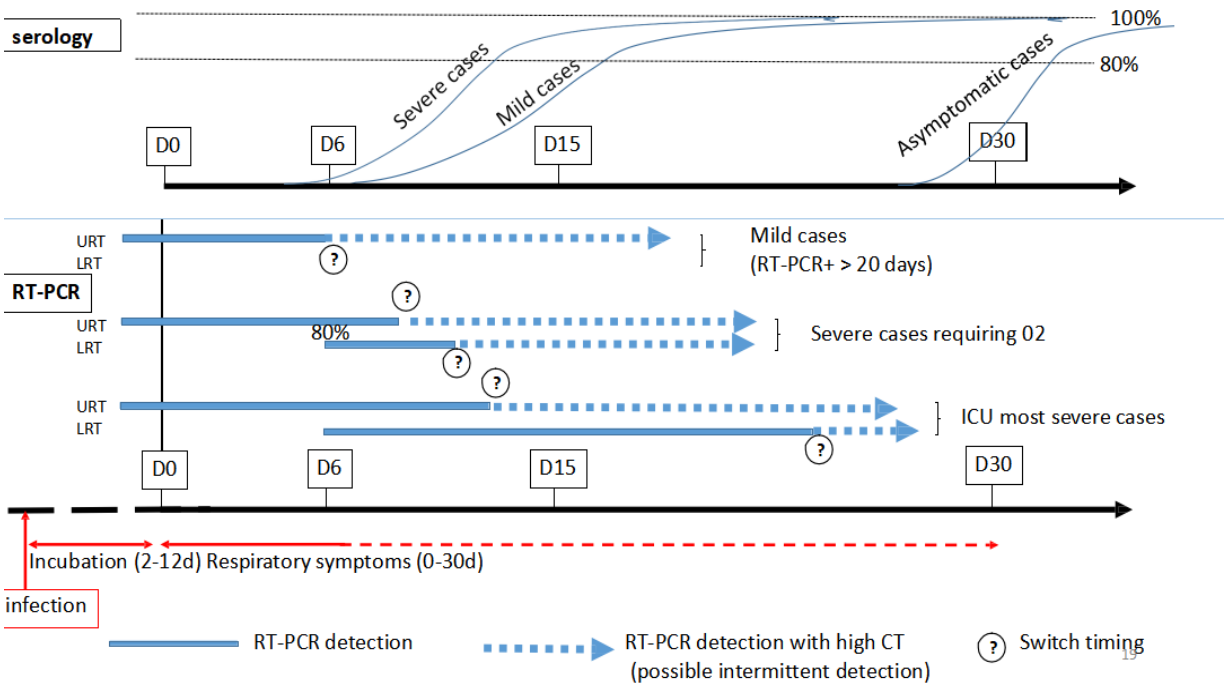


Figure 11. **SARS-CoV-2 diagnostic strategy at different stages of disease: a comprehensive overview**

Diagnosis of a viral infection (figure 11), such as COVID-19, can be made by detecting the presence of the virus or its genetic material using PCR (polymerase chain reaction) tests. These

tests can be done on samples taken from the upper respiratory tract (e.g., nasal swabs) or the lower respiratory tract (e.g., sputum samples). PCR tests can detect the virus even before the incubation period (i.e., the time between exposure and the onset of symptoms), during the incubation period, and before the onset of symptoms.

In severe cases of COVID-19, where patients require ICU care or supplemental oxygen, the virus may be detectable in respiratory samples for longer periods, sometimes up to 30 days or more. Additionally, the timing of seroconversion (the development of antibodies against the virus) may differ between different types of cases. Severe cases tend to seroconvert earlier than mild cases, while asymptomatic cases may seroconvert much later than mild or severe cases.

It's worth noting that while PCR tests are useful for detecting active infections, serology tests are used to detect past infections by measuring the presence of antibodies. Both types of tests are important in understanding the epidemiology of a disease and guiding public health interventions.

Since the first SARS-CoV-2 genome was published in January 2020, several real-time reverse-transcriptase polymerase chain reaction (RT-PCR) assays have been developed and widely implemented in clinical virology laboratories worldwide. These assays target different gene regions of the virus, including RdRp, N, E, ORF1ab nsp10, and ORF1b nsp14. The World Health Organization (WHO) has published several approaches developed by referral laboratories, including Charité (Germany), HKU (Hong Kong), China CDC (China), US CDC (United States), and Institut Pasteur, Paris (France).

Many tests and kits allow one to verify if a subject has been infected, from the most thorough and precise ones, such as molecular tests (RT-PCR, LAMP), to the more commercial, such as rapid antigenic or serological tests. The differences between the molecular test to the antigenic tests are that the molecular ones look for the genetic material of the virus, the serological look for the presence of an antibody response in progress, so the presence of IgG or IgM, and the antigenic look for structure materials of the virus, like Spike or nucleocapsid glycoproteins [90].

PCR is a widely used method for the diagnosis of COVID-19. This test detects the genetic material of the virus in a patient's respiratory specimen. A positive PCR test result confirms the diagnosis of COVID-19.

Rapid Antigen Test is similar to the PCR test but can provide results much faster, usually within 15-30 minutes. The rapid antigen test detects viral proteins in the respiratory specimen. This test may have a higher chance of false-negative results than PCR tests. An antibody test can detect the presence of antibodies in a person's blood that were produced in response to a SARS-CoV-2 infection. This test can be helpful in identifying people who were infected in the past and may have some immunity to the virus.

The gold standard for the diagnosis of COVID-19 lung disease remained nucleic acid amplification testing of SARSCoV-2 virus-specific sequences. Reverse transcription polymerase chain reaction (PCR) on nasopharyngeal swabs was the most commonly used technique for this purpose. However, the sensitivity of this test may vary depending on several factors, including the stage of the infection, sample collection, and processing.

SARS-CoV-2 serology tests, which detect antibodies produced by the immune system in response to the virus, can also aid in the diagnosis of COVID-19. These tests measure different antibody isotypes, including IgG, IgM, and IgA, and have shown a higher sensitivity than assays measuring only IgG. However, serology tests are not recommended for the initial diagnosis of COVID-19 due to the delay in antibody production and the potential for false-positive results.
During the peak infection rate, many healthcare systems faced a shortage of testing resources, including PCR capacity. This shortage left many patients with milder clinically suspected infections untested. In such situations, healthcare providers may have to rely on clinical symptoms and epidemiological factors, such as exposure to a confirmed case, to make a diagnosis and initiate appropriate care [92].

Role and challenges of SARS-CoV-2 serology

SARS-CoV-2 serology, involves the detection of antibodies against the virus in blood samples, can play an important role in several aspects related to the COVID-19 pandemic. It can be used to diagnose a current or past infection with SARS-CoV-2. Antibodies against the virus can be detected in blood samples a few days after symptom onset and can remain detectable for several months. Serological testing can help track the spread of SARS-CoV-2 in the population by identifying individuals who have been exposed to the virus, including those who may have had asymptomatic infections. This information can help public health officials determine how close a population is to achieving herd immunity, which is when enough individuals in a population are immune to a disease that it can no longer spread effectively. Furthermore, serology can help assess an individual's immune response to SARS-CoV-2 and determine if they have antibodies against SARS-CoV-2 and are therefore presumed to have some degree of immunity to the virus. Some countries have considered issuing "immunity passports" or similar documents to such individuals, which would allow them to travel or engage in other activities that are restricted for those who have not had COVID-19 or been vaccinated. This information can be useful for vaccine development and distribution, as well as for understanding the long-term effects of COVID-19. The longevity of immunity against SARS-CoV-2 is not yet well understood, and serology can help provide information about the duration of humoral (antibody-mediated) protective immunity following infection or vaccination. Serology can be used to screen potential plasma donors and

ensure that they have high levels of antibodies against SARS-CoV-2. Convalescent plasma therapy involves transfusing plasma (the liquid portion of blood that contains antibodies) from individuals who have recovered from COVID-19 into patients who are currently infected. Serological testing can be used to evaluate the effectiveness of COVID-19 vaccines by measuring the levels of antibodies produced by vaccinated individuals. This can help determine how well the vaccines are working and inform decisions about booster shots or modifications to the vaccines.

However, there are also several challenges associated with SARS-CoV-2 serology. These include:

Timing of testing: It can take several weeks after infection for antibodies against SARS-CoV-2 to develop, which means that serology may not be useful for diagnosing acute infections. Additionally, the timing of testing can affect the accuracy of serology results, as antibody levels may decline over time.

Variability in antibody response: Not all individuals who are infected with SARS-CoV-2 develop antibodies, and those who do may produce different types and levels of antibodies. This variability can make it difficult to interpret serology results and compare them across individuals or populations.

Lack of standardized testing: There are several different serological tests available for SARS-CoV-2, and they vary in their sensitivity and specificity. Standardization of testing protocols and validation of tests is needed to ensure that serology results are reliable and can be used to make informed decisions.

Detection of neutralising antibodies

Virus neutralisation assays are critical tools commonly used to determine whether antibodies present in a patient's serum or plasma can neutralize a virus in vitro, which means they can prevent the virus from infecting cells. In the case of SARS-CoV-2, neutralisation assays can provide important information about the effectiveness of COVID-19 vaccines or convalescent plasma in preventing viral infection [93].This information is important for determining the appropriate dosage of vaccines and therapeutic antibodies needed to achieve the desired level of protection.

In addition, virus neutralisation assays are useful for monitoring immune evasion by viral variants. As viruses mutate and evolve, they may develop new strains that are resistant to neutralisation by existing antibodies. Virus neutralisation assays can help identify viral variants that are resistant to neutralisation and guide the development of new vaccines and therapies.

Finally, virus neutralisation assays can provide valuable information about post-infection immunity. By measuring the levels of neutralising antibodies in individuals who have recovered

from viral infections, researchers can gain insights into the immune response to the virus and the duration of immunity.

To perform a neutralisation assay, the patient's serum or plasma is first incubated with live virus. Then, the virus-serum mixture is added to cultured cells, and the cells are incubated to allow for viral infection. The ability of the serum or plasma to neutralize the virus is determined by measuring the amount of virus replication in the cultured cells. Since SARS-CoV-2 is a highly infectious and potentially dangerous virus, neutralisation assays must be performed in a biosafety level 3 (BSL-3) laboratory [18].

There are **three types of neutralisation assays** that are commonly used:

Live virus neutralisation tests, such as the plaque reduction neutralisation assay (PRNT) [93] and microneutralisation assay [94], are considered the gold standard for measuring the ability of antibodies to neutralize SARS-CoV-2 virus. These assays use live, infectious SARS-CoV-2 virus obtained from a clinical isolate. In these assays, the serum or plasma sample is incubated with a fixed amount of live virus before being added to cultured cells. The neutralisation activity of the antibodies in the sample is then determined by measuring the inhibition of virus replication in the cells. The plaque reduction neutralisation assay and microneutralisation assay are performed in biosafety level 3 (BSL-3) laboratories because of the potential risk of handling live virus [18]. The results of live virus neutralisation assays can take approximately 5 days to obtain, as the assays require incubation of the virus and cells for a specific period of time to allow for virus replication and the development of visible plaques or cytopathic effects. PRNT is time-consuming and limited to 6- or 24-well plate format [94], however, this assay is highly specific and sensitive, and is considered the most reliable method for assessing the neutralising activity of antibodies against SARS-CoV-2. In addition to the PRNT and microneutralisation Test, the Focus Reduction Neutralisation Test (FRNT) is another type of neutralisation assay that can be used to evaluate the ability of antibodies to neutralize viruses. This test is similar to the PRNT, but instead of counting plaques, the number of viral foci (clusters of infected cells) is measured [94]. FRNT is high-throughput method based on PRNT. FRNT, it has a shorter process, uses a 96-well plate and requires less sample volume to quantify neutralising antibodies [93]. Like the PRNT and microneutralisation Test, the FRNT is also performed in biosafety level 3 (BSL-3) laboratories. However, the FRNT can be less sensitive than the PRNT and microneutralisation test, as the number of foci can be more variable than the number of plaques.

Surrogate virus neutralisation tests: In some cases, it may not be feasible or safe to use live virus in the neutralisation assay. In these cases, surrogate viral neutralisation tests (sVNT) that use a surrogate virus, such as a recombinant protein or a synthetic peptide, to mimic the behavior of the target virus. sVNT can be used to measure the levels of neutralising antibodies in patient

sera or vaccine recipients [94]. The serum or plasma sample is incubated with the surrogate virus before being added to cultured cells. The neutralisation activity of the antibodies in the sample is then determined by measuring the inhibition of virus replication in the cells. sVNT can be used to predict humoral protection and vaccine efficacy during clinical trials and after large-scale vaccination, the information regarding the biosafety level required for these tests is not entirely accurate.

While sVNT can be performed with a lower level of biosafety, such as biosafety level 1 or 2 facilities, the handling of clinical specimens and samples should still be performed following appropriate safety measures and protocols to prevent any potential exposure or contamination [18].

Pseudovirus neutralisation tests: Pseudoviruses are non-infectious particles that are engineered to express the surface proteins of the target virus onto the core of a different virus [26,27,93]. Pseudoviruses are chimeric viruses that can be used as a safer alternative to live virus in neutralisation assays and can be handled in biosafety level 2 (BSL-2) facilities [94]. In the case of SARS-CoV-2, the spike protein is incorporated onto the core of a different virus, such as Vesicular stomatitis virus (VSV) or human immunodeficiency virus type 1 (HIV-1) [96,97]. Pseudoviruses can be genetically modified to incorporate mutations that mimic emerging variants of SARS-CoV-2 [97].

Pseudovirus neutralisation assays have been developed using cell lines such as human ACE2 293T, human Calu-3, Vero-E6, Ips-CMs, and Huh-7 cells, which have shown substantial efficiency in detecting neutralising antibodies against SARS-CoV-2 [99,100]. Pseudovirus neutralisation assays can also use reporter genes, such as Gaussia luciferase (HIV-Gluc) or NanoLuciferase and GFP, to quantitatively measure the level of neutralisation [100].

Compared to the true virus, pseudotyped lentiviruses lack the genetic material of the corresponding infectious virus to which it is related and can only undergo a single infection cycle. The mechanism used by them to infect is similar to that of the true virus, but the latter otherwise can replicate multiple times. In addition to these advantages, the pseudotyped lentivirus system is quantifiable and can be produced rapidly [28].

1.5. Lentivirus system derived from HIV-1

Retroviral and lentiviral pseudotypes have been used as a safer alternative to replication-competent viruses for studying neutralising antibody responses to viral infections [102,103]. Lentiviral pseudotypes with the SARS-CoV spike protein have been used to investigate viral entry [103], identify receptors [104], examine viral tropism [105–107], and evaluate neutralising antibody responses [109–112]. Yang and colleagues (2004), in their research, developed lentiviral

pseudotypes with S, M, or E proteins and discovered that only the S protein was capable of facilitating viral entry into the designated target cells [106,112].

There are different systems used for producing lentiviral vectors (LVs), categorized into first, second, or third generation. All three systems involve the use of plasmids that enter the host cell and enable the expression of proteins necessary for virus assembly. First-generation LVs utilize three separate plasmids, including a Packaging Plasmid, an Envelope Plasmid, and a Transfer Plasmid. Second-generation LVs use the same approach as the first generation, but accessory proteins (Vif, Vpr, Vpu, and Nef) are removed from the Packaging Plasmid, leaving only gag, pol, rev, and Tat genes. Third-generation LVs use four plasmids, including a Packaging plasmid (gag and pol genes), a plasmid expressing the Rev protein, a plasmid coding for structural proteins, and a Transfer Plasmid. The HIV lentiviral vector contains the gag and pol genes necessary for constructing and assembling the pseudovirus. The gag gene encodes a polyprotein from which viral structural proteins p24, p17, p7, and p6 originate [114]. The pol gene is responsible for encoding a group of enzymes that are critical for the replication of HIV. These enzymes include reverse transcriptase (RT), protease, and integrase. In the replicative cycle of SARS-CoV-2, the virion is released from the endoplasmic reticulum (ER) to the Golgi apparatus, which results in the maturation and release of structural proteins from the host cell. In the case of a pseudotyped HIV lentivirus, synthesis occurs through the process of budding from the cell membrane, as illustrated in figure 12. This process is facilitated by the overexpression of Spike glycoprotein.

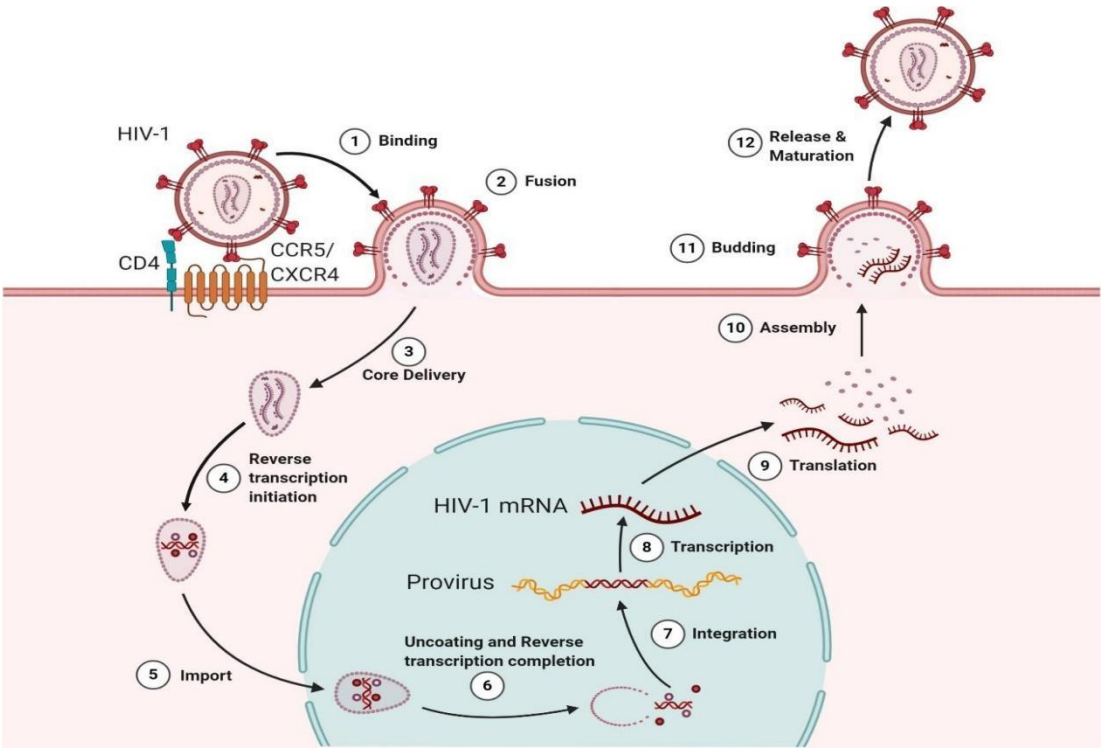


Figure 12. **HIV replication cycle** [114]

Conclusions: Testing is crucial in the fight against COVID-19. It helps identify infected individuals so that they can get appropriate care and prevent the further spread of the virus. Testing also helps public health officials understand the extent of the outbreak, monitor its spread, and make informed decisions about how to respond.

In addition, widespread testing is critical for reopening the economy safely. By identifying infected individuals, testing can help prevent outbreaks in workplaces, schools, and other settings. This can help prevent the need for more widespread shutdowns, which can have devastating economic and social consequences.

As new treatments and vaccines become available, testing will become even more critical. Testing will be necessary to identify individuals who are infected and need treatment, as well as to monitor the effectiveness of vaccines and track their distribution.

Overall, testing is a critical tool in the fight against COVID-19. By identifying infected individuals, testing can help save lives, prevent the spread of the virus, and enable us to safely reopen our economies and societies.

Testing is essential for tracking the spread of COVID-19 and evaluating the effectiveness of treatments and interventions. Furthermore, it allows healthcare providers to monitor patients' progress and adjust treatments as necessary, which can improve outcomes and save lives. Without testing, it would be difficult to determine whether a treatment is working or not.

In addition, testing is critical for understanding population immunity. By identifying individuals who have been exposed to the virus, public health officials can get a better understanding of how widespread the virus is and which populations may be at higher risk of infection. This information is crucial for planning and deploying vaccines.

It is also vital for identifying flare-ups and hotspots. By testing individuals in areas where there are suspected outbreaks, public health officials can quickly identify and contain the spread of the virus. This can help prevent widespread outbreaks and the need for more drastic measures, such as lockdowns.

Overall, testing is a crucial tool in the fight against COVID-19. It helps healthcare providers deliver effective treatments, enables the deployment of vaccines, and allows public health officials to track the spread of the virus and contain outbreaks.

2. MATERIALS AND METHODS

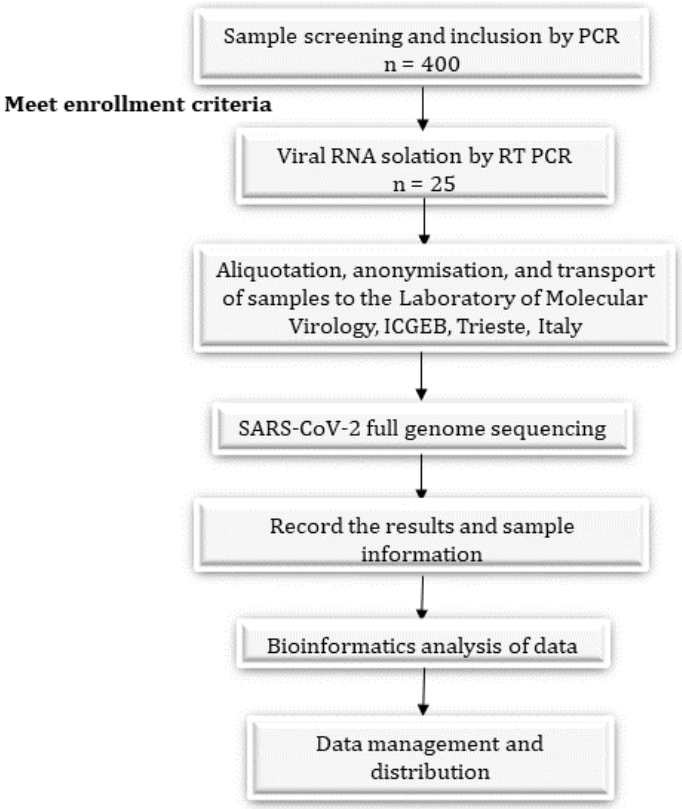
2.1. Study design

This study involved the whole genome sequencing of 25 strains of SARS-CoV-2 isolated from patients with different clinical forms and geographical regions from the Republic of Moldova. The SARS-CoV-2 RNA isolates were selected from the Alfa Diagnostica Laboratory Biobank based on a Commitment of Confidence. Viral RNA was isolated by RT PCR at Alfa Diagnostica Laboratory. For this, RT PCR reports were analysed and samples whose CT value was less than 30 were considered for inclusion in the study. Samples that were positive for the S gene and negative for the N gene were considered isolates with possible new mutations and with priority to be selected for sequencing. After the isolation of SARS-CoV-2 RNA, the samples were aliquoted and stored at -80°C. All samples were anonymized, and based on a Biological Materials Transfer Agreement, the samples were transported to the Molecular Virology Laboratory, ICGEB, Trieste, Italy, where the whole genome sequencing of the SARS-CoV-2 was performed. After obtaining the viral sequences, their quality was analysed, the viral genome was assembled, metadata was created, and the results obtained were uploaded to the GISAID international repository and NCBI. Also, the mutations of each isolated were analysed and a phylogenetic tree was created.

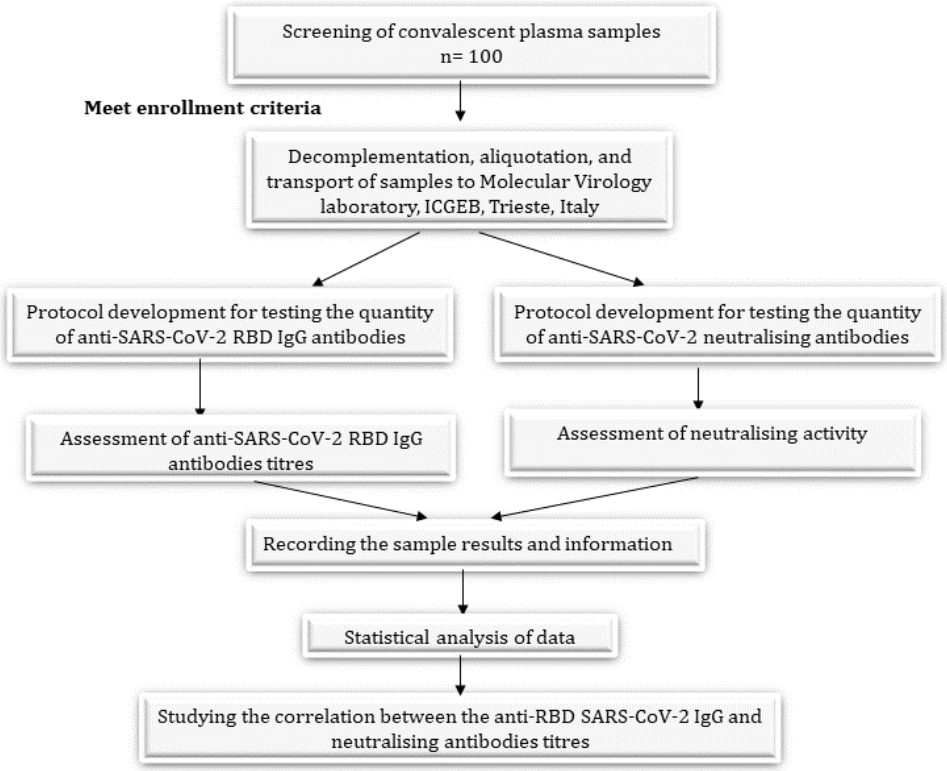
For the study of the immune response to infection, convalescent plasma from plasma donors, selected from the Biobank of the National Blood Transfusion Center, was included in the study. Furthermore, serum samples were taken from vaccinated patients. Personnel from Nicolae Testemitanu State University of Medicine and Pharmacy, including students and administrative staff, who wanted to receive the Sinopharm COVID-19 vaccine, were invited to participate in the study from March to May 2021. Participants completed an informed consent form and donated 10 millilitres of venous blood 14 days after receiving the second dose of the vaccine. Participants were queried regarding their demographic profile, prior COVID-19 infection history, and the timing of their second vaccine dose administration. Sera were separated from the collected blood and stored at -80ºC until use. All samples were anonymized and based on a Biological Materials Transfer Agreement; the samples were transported to the Molecular Virology Laboratory, ICGEB, Trieste, Italy. To assess the natural or vaccine-induced humoral response against COVID-19, sera were tested in duplicate for anti-RBD Spike IgG and neutralising antibodies using a home-made kit. All data were recorded and analysed.

2.2. Flow chart of cross-sectional study

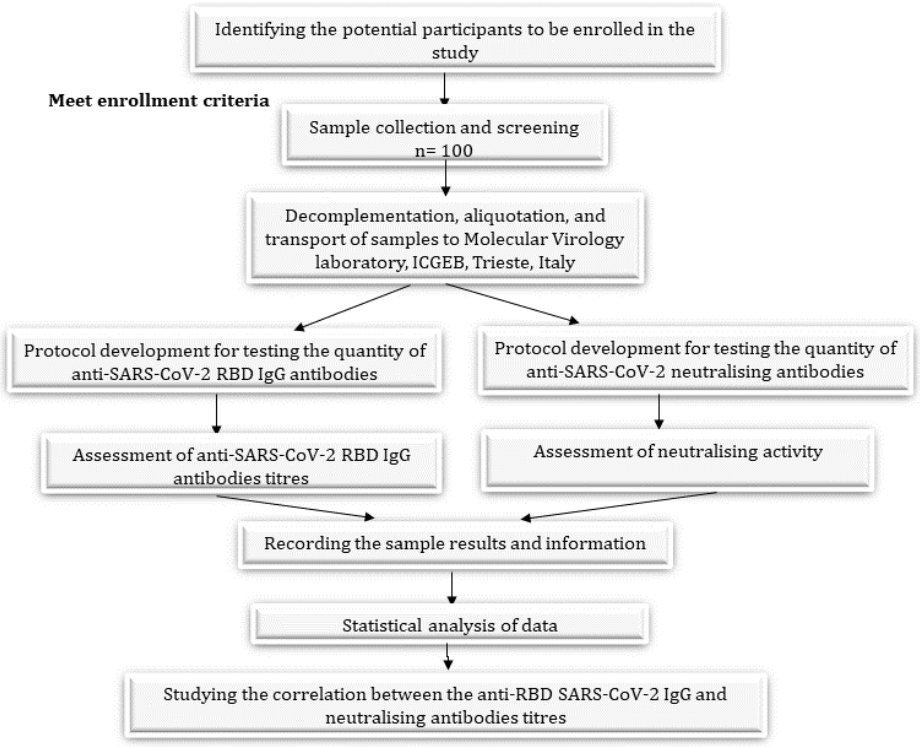
a) SARS-CoV-2 full genome sequencing



b) Studying the level of immunity among convalescent people



c) Studying the level of immunity among vaccinated individuals



2.3. Inclusion/exclusion criteria

a) SARS-CoV-2 full genome sequencing

- Inclusion: SARS-CoV-2 positive confirmed nasopharyngeal swabs by RT-PCR test with Ctvalue ≤ 30.

- Exclusion: insolates with the Ct value > 30.

b) Studying the level of immunity among convalescent and vaccinated people

- Inclusion:

Premium Plasma: Plasma collected from donors before 01.03.2020

Convalescent plasma: from patients with a negative result for COVID-19 in a PCR test and who are 14 days after clinical recovery. Age from 20 to 60 years, and detailed information about specimens is known.

Serum from vaccinated persons: Injections of both doses of the Sinopharm vaccine, were necessary for inclusion in this study. The person is 14 days after the Sinopharm vaccine booster, understands and signs the informed consent form, agrees to provide the medical history, and donate 10mL of venous blood, full data and information.

- Exclusion: positive COVID-19 test during the study period and an unwillingness to donate blood on schedule.

2.4. Information about specimens

Detailed information of specimens, including age (year), Sex, Date of symptoms onset, Date of vaccination (first dose and booster), Date of samples collection, Date of test, Type of specimen (nasopharyngeal swab, plasma, serum), PCR result, CT value, Clinical diagnosis, Diagnostic method, Locality.

3. GENOMIC VARIANTS AND PHYLOGENETIC ANALYSIS OF SARS-CoV-2 SEQUENCES FROM REPUBLIC OF MOLDOVA

Background

The COVID-19 pandemic, caused by the SARS-CoV-2 virus, has had a significant impact on global health and the world's economy since its emergence in late 2019. The virus spreads through respiratory droplets and close contact, leading to a wide range of clinical manifestations, from asymptomatic or mild to severe and even fatal cases. The speed of spread and the high number of cases in many countries led to overwhelmed healthcare systems, resulting in a massive burden on the health care workers.

As the virus continues to spread globally, there is a need to understand its transmission dynamics, clinical characteristics, and genomic evolution. Whole-genome sequencing (WGS) of SARS-CoV-2 has emerged as a powerful tool to track the spread and evolution of the virus. WGS allows for the identification of mutations and genetic changes that may affect viral transmissibility, virulence, and susceptibility to drugs and vaccines.

In the Republic of Moldova, the first SARS-CoV-2 case was reported on March 7th, 2020, and as of September 2021, there have been over 500,000 confirmed cases and more than 12,000 deaths. To understand the epidemiology and genetic diversity of the virus circulating in Moldova, a genomic surveillance program was established.

In this study, we sequenced 25 SARS-CoV-2 isolates from nasopharyngeal swabs collected from suspected cases meeting the Ministry of Health's case definition guidelines for SARS-CoV-2 infection. The samples were collected between June 2020 and September 2021 from different regions of Moldova, including the North, South, and Central parts of the country, from patients with various demographic data and different disease severity levels. SARS-CoV-2 genome sequencing was performed on samples meeting the epidemiological, clinical, and laboratory criteria.

Due to the lack of sequencing facilities in the country, international collaborations were necessary to perform the sequencing, highlighting the need for increased investment in sequencing facilities and expertise in low-resource countries to improve their capacity for real-time genomic surveillance of emerging infectious diseases.

This study aimed to characterize the genomic diversity of SARS-CoV-2 circulating in Moldova, identify the temporal and geographic distribution of SARS-CoV-2 strains in the country, and to provide insight into the transmission dynamics and evolution of the virus in the region.

Overall, the results of this study provide important information on the genetic diversity and evolution of SARS-CoV-2 in Moldova, which can inform public health interventions and guide

the development of effective control measures. The study also highlights the importance of international collaborations and investment in sequencing facilities and expertise in low-resource countries to improve their capacity for real-time genomic surveillance of emerging infectious diseases.

3.1. Materials and methods

3.1.1. Sample collection and selection

In this study, 25 SARS-CoV-2 isolates were sequenced from nasopharyngeal swabs collected from suspected cases that met the Ministry of Health's case definition guidelines for SARS-CoV-2 infection [23] between June 2020 and September 2021. To ensure participant confidentiality, all samples were codified. Throughout the duration of the study, all positive nasopharyngeal swabs from humans were stored at -80 °C.

However, due to the lack of sequencing facilities in the country, the initial strategy adopted was to partner with international institutions such as the International Centre for Genetic Engineering and Biotechnology (ICGEB) to carry out the sequencing. According to WHO recommendations [7] the rationale criteria for samples selection for sequencing depends on the epidemiological situation, target chosen and available resources. In countries with limited resources and capacity, such as Republic of Moldova, targeted sampling was found to be the most suitable approach. At the beginning of the pandemic when the country had no available resources to sequence the SARS-CoV-2, samples were collected from different outbreak areas, including the North, South, and Central parts of the country, from patients with various demographic data and different disease severity levels. SARS-CoV-2 genome sequencing was performed at the meeting of epidemiological, clinical, and laboratory criteria. This allowed us to obtain a diverse set of samples from different geographical areas and periods, which may help to increase the representativeness of the samples and the generalizability of the findings. However, the reliance on international partnerships highlights the need for increased investment in sequencing facilities and expertise in low-resource countries to improve their capacity for real-time genomic surveillance of emerging infectious diseases.

3.1.2. Extraction of total nucleic acids and qRT-PCR

Detection of SARS-CoV-2 was carried out at the *Nicolae Testemitanu* State University and Pharmacy from the Republic of Moldova in collaboration with Alfa Diagnostica Laboratory. The nasopharyngeal swabs were collected in a total volume of 1000 µL transport media (*Vector Best* REF: C-8885). An DNA/RNA manual extraction kit was used to extract the nucleic acid from clinical samples (RealBest extraction 100 *Vector Best* REF: C-8896*).* After resuspending the

pellets in 200 µL of eluting solution and incubating them at 65 °C for five minutes in a thermoshaker (BioSan Thermo-Shaker TS 100, SN: 01012019080450), the mixture was centrifuged for one minute at 13000 rpm before being placed in the magnet holder. The SARS-CoV-2/SARS-CoV Multiplex Real-Time PCR Detection Kit (DNA-Technology Research & Production, R3-P436-23/9EU) was used to confirm the diagnosis, with primers targeting the E-gene and N-gene. For amplification, a C1000 Touch Thermal Cycler CFX96 Real-Time System (Biorad) was used. For full genome sequencing, samples having a cycle threshold (CT) value under 30 were taken into consideration. The extracted SARS-CoV-2 RNA from selected samples was aliquoted, anonymized and based on a Material Transfer Agreement were sent at -80°C to International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy [10].

3.1.3. SARS-CoV-2 full-genome sequencing

The sequencing and data processing were carried out at the AREA Science Park's ARGO Open Lab Platform for Genome Sequencing and the ICGEB in Trieste, Italy. The conventional procedure for paired-end 150-bp reads was used for high-throughput sequencing on an Illumina MiSeq sequencer.

The raw sequence data were quality controlled using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The Primerclip trimming tool and adapter sequences from Swift Biosciences Accel-Amplicon panels were used for adapter removal and read trimming. Genome assembly was carried out employing Swift dockerized data analysis techniques [115,116]. All tools were executed with the default settings.

3.1.4. Library preparation and genome assembly

The RNA quantity and quality assessments, high-throughput sequencing library preparation, and raw sequence data quality control were all carried out as previously described [116–119]. The genome assembly was carried out in accordance with the standards [121,122]. To summarize, RNA quantities and quality were assessed using the Qubit 2.0 fluorimeter (Thermo Fisher Scientific, USA) and the Agilent 2100 bioanalyzer (Agilent Technologies, USA). Swift Amplicon SARS-CoV-2 research panel was used to analyze 100 ng of RNA (Swift Bioscience, USA) [10].

3.1.5. Data deposition

The 19 samples' information and coding full genome sequences were uploaded to the GISAID database (www.gisaid.org), where they can be accessed using the accession numbers shown in table 1. Moreover, sequences can be found in the NCBI database under the BioProject

ID PRJNA786454. The SRA accession numbers for each sample are shown in table 1 along with the raw reads that were submitted to the NCBI Sequence Read Archive (SRA) database [10].

3.1.6. Phylogenomic analysis

Phylogenetic analyses were performed through the Nextstrain bioinformatics platform [122]. A Moldova-focused country-level subsampling strategy was used in the context of the Nextregions/Europe data set (updated to 1 September 2021); the reference strain hCoV-19/Wuhan/WH01/2019 (GISAID accession number EPI_ISL_402125) was used as the original root [10].

The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. Maximum composite likelihood (MCL) approach was used, and the topology with a superior log likelihood value was selected. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.7827)). The rate variation model allowed some sites to be evolutionarily invariable ([+I], 68.58% sites). The analysis involved 476 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. A total of 3309 positions were included in the final dataset. The evolutionary analyses were conducted using MEGA7 [123]. Multiple sequence alignment was performed with the ClustalO software [124–126]. The alignment was trimmed to the spike gene coding region. Based on the Akaike information criterion the best evolutionary model was General Time Reversible G+I. Using this model, maximum likelihood trees were constructed using the MEGA 7 software. As a measure of the robustness of each node, the bootstrapping method was employed. The phylogenetic tree was edited using the FigTree program v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

3.2. Results and discussions

Altogether, the study obtained 19 whole-genome sequences from SARS-CoV-2-positive samples collected from humans in the Republic of Moldova between June 2020 and September 2021. The mean read depth for the sequences ranged from 80 to 200, and the sequences were ≈ 29,800 nucleotides in length. The genomic length of the samples was 29,541, with a 95% CI ranging from 28,673 to 30,080. The median genome size was 29,541, and its 95% CI was 29,540 to 29,542. The standard deviation of genome size was 1941. The IQR was 74.5, and the range of genome sizes observed was from 21,179 to 29,990 nucleotides. The mean coverage depth was 7,834x, with a 95% CI ranging from 7,519x to 8,255x. The median coverage depth was 8,149x, and its 95% CI was 8,149x to 8,149x (indicating no variation). The standard deviation of coverage depth was 854, and the IQR was 0. The range of coverage depth values observed was from 5,312x

to 8,149xas determined in GISAID (SAMtools v1.9) [128]. The mean GC content was 45.7%, with a 95% confidence interval (CI) of 45.5% to 45.9%. The median GC content was 45.7%, also with a 95% CI of 45.5% to 45.9%. The standard deviation (SD) of the GC content was 0.496. The interquartile range (IQR) was 0.55, and the range of values observed was from 44.8% to 46.6%. Sequencing parameters were determined for each sample and are shown in table 1 [10].

Initially, more than 2,900,000 complete SARS CoV2 virus genomes were downloaded from the GISAID database filtered by European geographic location [128]. Using home-made scripts, we filtered out all genomes containing undefined nucleotides (NNNs). Since the dataset was still large, we performed random sampling to reduce the number of sequences without losing population variability. The final dataset contains a total of 542 nucleotide sequences from several European countries. Of these 542 sequences, 19 correspond to the samples analysed in this study (EPI_ISL_516934, EPI_ISL_516935, EPI_ISL_516938, EPI_ISL_516936, EPI_ISL_3886549, EPI_ISL_3886552, EPI_ISL_3886554, EPI_ISL_3886554, EPI_ISL_ISL_3886556, EPI_ISL_3886557, EPI_ISL_3886560, EPI_ISL_3886562, EPI_ISL_3886564, EPI_ISL_3886566, EPI_ISL_3886568, EPI_ISL_3903715, EPI_ISL_3903716, EPI_ISL_3903717, EPI_ISL_3903718, EPI_ISL_3903719) (table 1).

The whole data set corresponds to viruses circulating between 2020 and 2022 in different European countries, including sequences from Moldova obtained in other studies. We decided to focus the phylogenetic analysis on the analysis of the genomic region corresponding to the spike protein gene because given that this protein is one of the main antigenic determinants of this virus. Multiple sequence alignment was performed with the ClustalO program implemented as command line software [124–126]. Once obtained, the alignment was trimmed to the spike gene coding region corresponding to the spike gene. Poorly aligned positions and divergent regions of ambiguous alignment were removed using the software GBlocks v.0.91b. [129,130]. Once alignment was correct, the best evolutionary model that described our sequence dataset was assessed using the “Find Model” interface in the Mega7 package. Based on several descriptors such as the Akaike information criterion (AIC), the Bayesian Information Criterion (BIC) and the Maximum Likelihood value (lnL) the best evolutionary model was General Time Reversible G+I. Using this model, maximum likelihood trees were constructed using the MEGA 7 software. As a measure of the robustness of each node, the bootstrapping method was employed. The phylogenetic tree was edited using the FigTree program v1.4.2, which is available online (http://tree.bio.ed.ac.uk/software/figtree/).

3.2.1. Phylogenetic analysis

In order to identify the circulating genotype of SARS-CoV-2 in Moldova, the sequences obtained in this study were compared to 542 complete SARS-CoV-2 genome sequences available in the Global Initiative on Sharing All Influenza Data (GISAID - Initiative) (as of May 26th 2022). Interestingly, samples collected in 2021 were identified as the Delta variant, while samples collected in 2022 were identified as the Omicron variant of SARS-CoV-2.

To get a very general idea of which SARS-CoV-2 variants have circulated in Moldova we proceeded to study a general picture of the phylogenetic relationships between the circulating viral cases in Moldova and the main SARS-CoV-2 lineages.

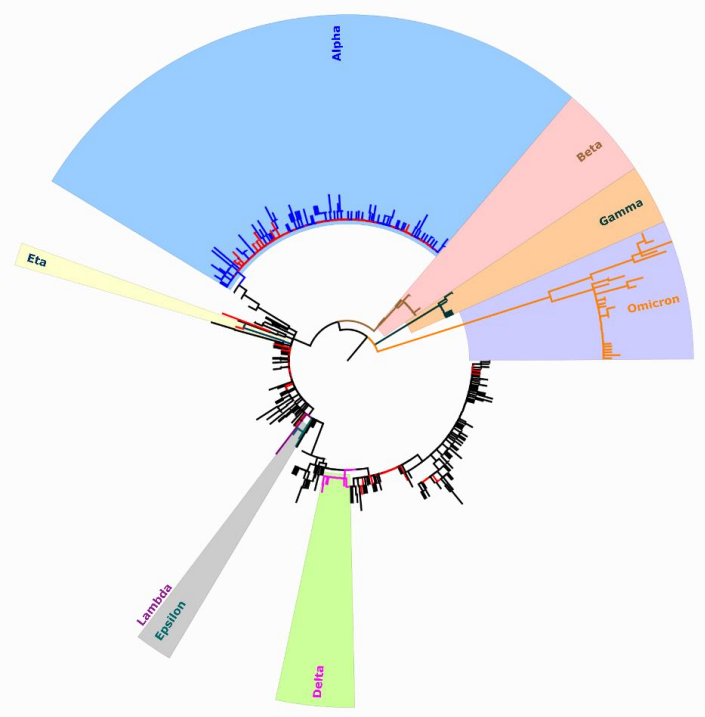


Figure 13. **Maximum-likelihood phylogenetic tree analysis of the spike gene of SARS-CoV2 circulating in Moldova during COVID19 pandemic.**

Looking in an anti-clockwise direction, Delta is highlighted in green, Epsilon (grey), Lambda (magenta), Eta (yellow), Alpha (sky-blue), Beta (pink), Gamma (orange) and Omicron (lilac). The branches of the tree corresponding to sequences from different European countries are coloured in black. Moldova's circulating sequences are highlighted in red.

The main SARS-CoV-2 lineages are highlighted in the phylogenetic tree (figure 13). As can be seen in the figure, the Moldova strains (painted in red) cluster mainly with strains from the Alpha lineage, denoting a close genetic relationship between these sequences. However, the phylogeny also shows somewhat close genetic relationships between circulating variants in

Moldova and sequences from the Eta, Lambda and Delta lineages. On the other hand, no phylogenetic relationship was observed between the Moldovan Strains and the Beta, Gamma and Omicron lineages.

With these results it could be said that the SARS CoV-2 viruses that have circulated in Moldova are genetically related to a greater extent to sequences of the Alpha lineage. However, we also found some genetic linkage of the Eta, Lambda and Delta lineages with circulating viruses in Moldova. This denotes the great variability of viral variants that have been circulating in Moldova. This result was expected as pandemic events of this style are characterized by rapid evolution where many viral variants are generated.

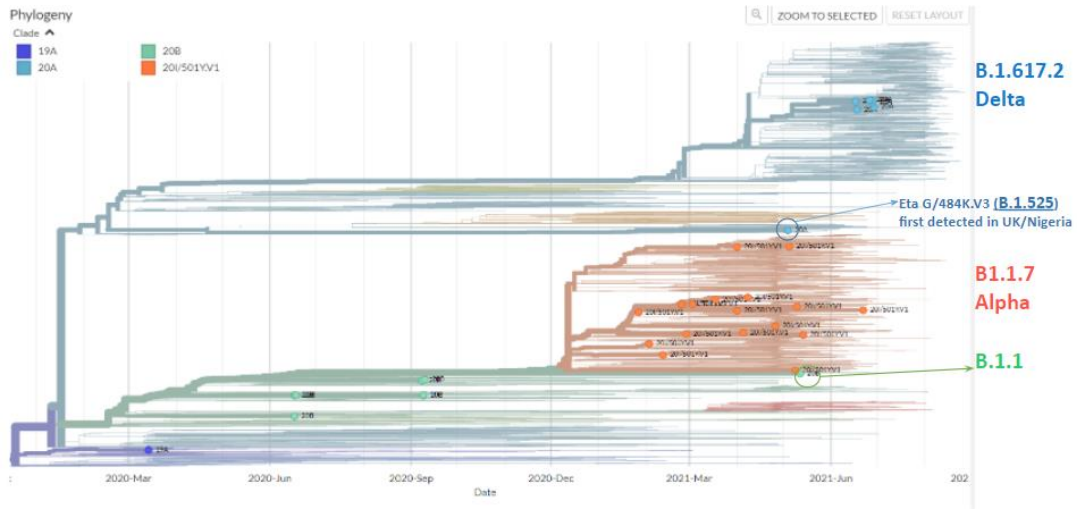


Figure 14. **Genomic evolution of SARS-CoV-2 in Republic of Moldova** [10]

The phylogenetic analysis showed that the Moldovan isolates were clustered together and shared a common ancestor with other European SARS-CoV-2 isolates, indicating the introduction of SARS-CoV-2 into Republic of Moldova from multiple sources. To get a deeper insight into genetic relationships we should do similar analyses but including the whole genome as lineage classification takes into account several genomes feature not only the spike gene.

To understand the virus's evolutionary history in this region we also performed a phylogenetic analysis using Nextstrain bioinformatics platform, by which 5 clades were identified viz. 19A, 20A, 20B, 20I/501Y.V1 and 21D from March 2020 until September 2021 (table 1, figure 14). Figure 14 shows the genetic relationship between Moldova complete genome sequences in the context of the Nextregions/Europe data set (updated to September 1st, 2021). A Moldova Republic of - focused country-level subsampling strategy was performed, using the reference strain hCoV-19/Wuhan/WH01/2019 (GISAID accession number EPI_ISL_402125) as the original root [10].

Table 1. Genomic characteristics of SARS-CoV-2 isolates from nasopharyngeal swabs in the Republic of Moldova [10]

N/o	VIRUS ISOLATE	GISAID Accession ID	PATIENT location	SRA Library ID	Collection	Submission	GISAID Clade	Nextstrain Clade	No. of raw reads	Genome Size (bp)	SARS-CoV-2 Coverage depth	GC content	Pango lineage	Amino acid substitutions
1	hCoV-19/Moldova/ICGEB_MD6/2020	EPI_ISL_516938 2020-06-17	Moldova/ Rabnita	**SRX14472462**	18/06/2020	24/08/2020	GR	20B	1111830	29.900	5,570x	45.20%	B.1.1 (Pango v.3.1.20 2022-02-28)	Spike D614G, N G204R, N R203K, NS3 S165F, NSP2 L410F, NSP3 N1785D
2	hCoV-19/Moldova/ICGEB_MD7/2020	EPI_ISL_516936 2020-06-17	Moldova/ Straseni	**SRX14472461**	18/06/2020	24/08/2020	GR	20B	1050968	29.886	8,149x	45.00%	B.1.1 (Pango v.3.1.20 2022-02-28)	
3	hCoV-19/Moldova/ICGEB_MD4/2020	EPI_ISL_516935 2020-06-18	Moldova/ Balti	**SRX14472460**	17/06/2020	25/08/2020	G	20A	1080708	21.179	5,312x	45.50%	B.1	
4	hCoV-19/Moldova/ICGEB_MD1/2020	EPI_ISL_516934 2020-06-18	Moldova/ Chisinau	**SRX14472459**	17/06/2020	26/08/2020	GR	20B	932600	29.867	7,583x	45.50%	B.1.1 (Pango v.3.1.20 2022-02-28)	
5	hCoV-19/Moldova/ICGEB_818767_S16/202	EPI_ISL_3886568	Moldova/ Cimislia	**SRX13323315**	11/05/2021	02/09/2021	GR	20I/501Y.V1	929928	29,540	8,149x	45.30%	B.1.1.7 (Pango v.3.1.20 2022-02-28), Alpha (B.1.1.7-	Spike A570D, Spike D614G, Spike D1118H, Spike P681H, Spike S982A, Spike T716I, N D3L, N G204R,

													like) (Scorpio)	N R203K, N S235F, NS3 D27Y, NS8 Q27stop, NS8 R52I, NS8 Y73C, NSP3 A890D, NSP3 I1412T, NSP3 T183I, NSP6 G188C, NSP12 P323L, NSP12 V405A
6	hCoV-19/Moldova/ICGEB_819913_S18/2021	EPI_ISL_3903719	Moldova/ Chisinau	SRX13323314	12/05/2021	03/09/2021	GR	20I/501Y.V1	513580	29,542	8,149x	45.80%	B.1.1.7 (Pango v.3.1.20 2022-02-28), Alpha (B.1.1.7-like) (Scorpio)	Spike A570D, Spike D614G, Spike D1118H, Spike N501Y, Spike P681H, Spike S982A, Spike T716I, Spike V445I, N D3L, N G204R, N R203K, N S235F, NS7a P84A, NS8 Q27stop, NS8 R52I, NS8 Y73C, NSP2 R362H, NSP3 A890D, NSP3 D410G, NSP3 I1412T, NSP3 T183I, NSP12 P323L

7	hCoV-19/Moldova/ICGEB_809515_S10/2021	EPI_ISL_3886566	Moldova/ Chisinau	**SRX13323313**	05/05/2021	04/09/2021	GR	20I/501Y.V1	435738	29,540	8,149x	45.90%	B.1.1.7 (Pango v.3.1.20 2022-02-28), Alpha (B.1.1.7-like) (Scorpio)	Spike A570D, Spike D614G, Spike D1118H, Spike P681H, Spike S982A, Spike T716I, N D3L, N G204R, N R203K, N S235F, NS8 Q27stop, NS8 R52I, NS8 Y73C, NSP3 I1412T, NSP3 T183I, NSP5 Y101C, NSP12 P323L, NSP13 T115I
8	hCoV-19/Moldova/ICGEB_812387_S15/2021	EPI_ISL_3886564	Moldova/ Dubasari	**SRX13323312**	07/05/2021	05/09/2021	GR	20I/501Y.V1	551564	29,541	8,149x	46.10%	B.1.1.7 (Pango v.3.1.20 2022-02-28), Alpha (B.1.1.7-like) (Scorpio)	Spike A570D, Spike D614G, Spike D1118H, Spike N501Y, Spike P681H, Spike S982A, Spike T716I, N D3L, N G204R, N R203K, N S235F, NS8 Q27stop, NS8 R52I, NS8 Y73C, NSP3 A890D, NSP3 I1412T, NSP3 T183I, NSP12 P323L

9	hCoV-19/Moldova/ICGEB_819835_S17/2021	EPI_ISL_3886562	Moldova/ Causeni	**SRX13323311**	12/05/2021	06/09/2021	GR		688252	29,541	8,149x	46.40%	B.1.1.523 (Pango v.3.1.20 2022-02-28)	Spike D614G, Spike D839V, Spike E780A, Spike F306L, Spike N211K, Spike T1027I, M I82T, N D22Y, N G204R, N G212C, N R203K, NSP2 N269D, NSP3 F1496L, NSP3 M84V, NSP3 R1297I, NSP4 T114I, NSP5 V303I, NSP6 V84F, NSP10 T111I, NSP12 P323L, NSP12 S229N, NSP13 L455M, NSP13 P77L, NSP15 V338A
10	hCoV-19/Moldova/ICGEB_797186_S3/2021	EPI_ISL_3886560	Moldova/ Chisinau	**SRX13323323**	24/04/2021	07/09/2021	GR	20I/501Y.V1	608708	29,541	8,149x	45%	B.1.1.7 (Pango v.3.1.20 2022-02-28), Alpha (B.1.1.7-like) (Scorpio)	Spike A570D, Spike D614G, Spike D1118H, Spike P681H, Spike S982A, Spike T716I, N D3L, N G204R, N R203K, N S235F, NS3 I232V, NS8

														Q27stop, NS8 R52I, NS8 Y73C, NSP3 A338T, NSP3 A890D, NSP3 I1412T, NSP3 T183I, NSP12 P323L
11	hCoV-19/Moldova/ICGEB_807867_S7/2021	EPI_ISL_3903718	Moldova/ Chisinau	**SRX13323322**	05/05/2021	08/09/2021	GR	20I/501Y.V1	804896	29,542	8,149x	46.40%	B.1.1.7 (Pango v.3.1.20 2022-02-28), Alpha (B.1.1.7-like) (Scorpio)	Spike A570D, Spike D614G, Spike D1118H, Spike N501Y, Spike P681H, Spike S982A, Spike T716I, N G204R, N R203K, N S235F, NS8 Q27stop, NS8 R52I, NS8 Y73C, NSP3 A890D, NSP3 G908D, NSP3 I1412T, NSP3 T183I, NSP12 P323L
12	hCoV-19/Moldova/ICGEB_807539_S5/2021	EPI_ISL_3903717	Moldova/ Chisinau	**SRX13323321**	04/05/2021	09/09/2021	G	21D	905208	29,464	8,149x	45.80%	(Pango v.3.1.20 2022-02-28), Eta (B.1.525-like) (Scorpio)	Spike A67V, Spike D215Y, Spike D614G, Spike E484K, Spike F888L, Spike Q52R, Spike Q677H, E L21F, M I82T,

														N A12G, N T205I, NSP3 D1225G, NSP3 G255V, NSP3 T1189I, NSP5 P96L, NSP12 P323F, NSP12 V405A
13	hCoV-19/Moldova/ICGEB_823299_S20/2021	EPI_ISL_3886557	Moldova/ Stefan Voda	**SRX13323320**	14/05/2021	10/09/2021	GR	20I/501 Y.V1	817382	29,541	8,149x	45.50%	B.1.1.7 (Pango v.3.1.20 2022-02-28), Alpha (B.1.1.7-like) (Scorpio)	Spike A570D, Spike D614G, Spike D1118H, Spike G1219C, Spike P681H, Spike S982A, Spike T716I, N D3L, N G204R, N R203K, N S235F, NS8 Q27stop, NS8 R52I, NS8 Y73C, NSP3 A890D, NSP3 I1412T, NSP3 T183I, NSP6 A51V, NSP12 P323L
14	hCoV-19/Moldova/ICGEB_810217_S12/2021	EPI_ISL_3886556	Moldova/ Chisinau	**SRX13323319**	05/05/2021	11/09/2021	GR	20I/501 Y.V1	518006	29,534	8,149x	46%	B.1.1.7 (Pango v.3.1.20 2022-02-28), Alpha (B.1.1.7-	Spike A570D, Spike D614G, Spike D1118H, Spike H69del, Spike P681H, Spike S982A, Spike T716I, Spike V70del,

													like) (Scorpio)	N D3L, N G204R, N R203K, N S235F, NS8 Q27stop, NS8 R52I, NS8 Y73C, NSP3 A890D, NSP3 I1412T, NSP3 T183I, NSP9 T19I, NSP12 P323L
15	hCoV-19/Moldova/ICGEB_797065_S2/2021	EPI_ISL_3886554	Moldova/ Chisinau	**SRX13323318**	24/04/2021	12/09/2021	GR	20I/501 Y.V1	553028	29,540	8,149x	45.60%	B.1.1.7 (Pango v.3.1.20 2022-02-28), Alpha (B.1.1.7-like) (Scorpio)	Spike A570D, Spike D614G, Spike D1118H, Spike P681H, Spike S982A, Spike T716I, N D3L, N G204R, N R203K, N S235F, NS3 T271I, NS7b F9S, NS8 Q27stop, NS8 R52I, NS8 Y73C, NSP3 I1412T, NSP3 T183I, NSP12 P323L
16	hCoV-19/Moldova/ICGEB_810376_S13/2021	EPI_ISL_3903716	Moldova/ Chisinau	**SRX13323317**	05/05/2021	13/09/2021	G	20I/501 Y.V1	772108	29,537	8,149x	45.70%	B.1.1.7 (Pango v.3.1.20 2022-02-28),	Spike A570D, Spike D614G, Spike D1118H, Spike P681H, Spike S982A,

													Alpha (B.1.1.7-like) (Scorpio)	Spike T307I, Spike T716I, N D3L, N R203K, N S235F, NS8 K68stop, NS8 Q27stop, NS8 R52I, NS8 Y73C, NSP2 C475R, NSP3 A890D, NSP3 I1412T, NSP3 T183I, NSP12 E802D, NSP12 P323L, NSP13 D450N, NSP14 S418C
17	hCoV-19/Moldova/ICGEB_816078_S14/2021	EPI_ISL_3903715	Moldova/ Chisinau	**SRX13323316**	09/05/2021	14/09/2021	GR	20I/501Y.V1	819352	29,687	8,149x	46.60%	B.1.1.7 (Pango v.3.1.20 2022-02-28), Alpha (B.1.1.7-like) (Scorpio)	Spike A570D, Spike D614G, Spike D1118H, Spike N501Y, Spike P681H, Spike S982A, Spike T716I, N A55S, N G204R, N R203K, N S235F, NS8 Q27stop, NS8 R52I, NS8 Y73C, NSP3 A890D, NSP3 I1412T, NSP3 T183I, NSP12

														P323L, NSP15 N74S
18	hCoV-19/Moldova/ICGEB_798119_S4/2021	EPI_ISL_3886552	Moldova/ Chisinau	**SRX13323310**	26/04/2021	15/09/2021	GR	20I/501Y.V1	995082	29,689	8,149x	44.80%	B.1.1.7 (Pango v.3.1.20 2022-02-28), Alpha (B.1.1.7-like) (Scorpio)	Spike A570D, Spike D614G, Spike D1118H, Spike P631S, Spike P681H, Spike S982A, Spike T716I, Spike W152R, N D3L, N G204R, N R203K, N S235F, NS8 Q27stop, NS8 R52I, NS8 Y73C, NSP3 A890D, NSP3 I1412T, NSP3 S1717L, NSP3 T183I, NSP12 P323L, NSP14 K165R, NSP16 P236L
19	hCoV-19/Moldova/ICGEB_796301_S1/2021	EPI_ISL_3886549	Moldova/ Hincesti	**SRX13323309**	24/04/2021	16/09/2021	GR	20I/501Y.V1	621908	29,541	8,149x	45.80%	B.1.1.7 (Pango v.3.1.20 2022-02-28), Alpha (B.1.1.7-like) (Scorpio)	Spike A570D, Spike D614G, Spike D1118H, Spike P681H, Spike S982A, Spike T716I, N D3L, N G204R, N R203K, N S235F, NS7a P34S, NS8

														Q27stop, NS8 R52I, NS8 Y73C, NSP2 L550F, NSP2 S203G, NSP3 A890D, NSP3 I1412T, NSP3 T183I, NSP3 V473F, NSP6 L37F, NSP8 T187I, NSP12 P323L, NSP16 D179G

At the beginning of the pandemic the viral diversity was low in the Republic of Moldova. In March 2020 19A Nextrain clade was circulating in the country. Between March 2020 to September 2020 the country was in the lockdown and the viral diversity was still low but was replaced by clade 20B. Later, multiple introductions of SARS-CoV-2 lineages B.1.1., B.1.1.7 and B.1.1.525 were detected in the Country mainly through Chisinau International Airport. B.1.1.7 lineage became predominant between December and June 2021. Then in June 2021 Delta variant began to increase in number.

Several amino acid substitutions were detected in the 19 SARS-CoV-2 samples collected from Moldova and analysed in this study. These substitutions were found in the spike protein, nucleocapsid protein, non-structural proteins (NS3, NS8, NSP2, NSP3, NSP5, NSP6, NSP7a, NSP9, NSP10, NSP12, NSP13, NSP14, and NSP15), and envelope protein (E). The substitutions in the spike protein included: Spike D614G, Spike A570D, Spike D1118H, Spike P681H, Spike S982A, Spike T716I, Spike V445I, Spike N501Y, Spike E484K, and Spike F888L, among others. These substitutions are known to have varying impacts on the infectivity and virulence of SARS-CoV-2, and their presence in the samples collected from Moldova suggests that the virus is evolving in this region.

In addition to the Spike protein, other proteins also exhibited amino acid substitutions in the Moldovan samples, including Nucleocapsid (N), Non-Structural Protein 3 (NSP3), RNA-dependent RNA polymerase (NSP12) and in the envelope protein (E). The substitutions in the nucleocapsid protein included G204R, R203K, and S235F, while those in the non-structural proteins included D27Y, Q27stop, R52I, Y73C, A890D, I1412T, T183I, G908D, and D410G. The substitutions detected in the envelope protein included L21F, I82T, and T205I. These substitutions are also known to have functional significance and may impact the transmissibility and virulence of SARS-CoV-2.

Spike D614G: This is a substitution of aspartic acid (D) to glycine (G) at position 614 in the Spike protein. It is a well-known mutation that was first detected in Germany and China in late January 2020 [131]. It was noted to be increasing in frequency in April 2020 and became the most widespread variant of SARS-CoV-2 worldwide [132–134]. This mutation was noted in 540 out of 542 SARS-CoV-2 sequences from the Republic of Moldova.

Another mutation located on the RBD is N501Y, common to all SARS-CoV-2 variants except the Delta variant [135]. This mutation was found in 216 SARS-CoV-2 isolates out of 542 Republic of Moldova sequences uploaded in GISAID, from which four samples were analysed in this paper (EPI_ISL_3903719, EPI_ISL_3886564, EPI_ISL_3903718, EPI_ISL_3903715). N501Y mutation is of particular concern for increasing the affinity between RBD and ACE2 [136] and was noted first in Republic of Moldova in January 2021.

In one sample (EPI_ISL_3903717) collected in May 2021 was detected E484K mutation. This mutation is also in the RBD of S and is associated with immune escape and increased ACE2 affinity [137]. The Iota variant possesses the E484K mutation that is associated with evading antibodies found in the Beta and Gamma variants. This indicates that it may also diminish the efficacy of existing vaccines in a similar manner [138].

The L452R mutation is a substitution of the amino acid leucine (L) to arginine (R) at position 452 in the Spike protein of SARS-CoV-2. This mutation has been identified in some isolates of the virus and has been associated with increased transmissibility and potential immune escape. This mutation is noted to have significant free energy changes [139]. The fact that 31.36% of the isolates (from June 2021 to January 2022) had this mutation suggests that this variant may have been circulating widely in the population during that time period. The prevalence of this mutation may vary depending on the location and time frame of the study, as well as other factors such as the effectiveness of surveillance systems.

The Eta variant detected in May 2021 harbour Q677H Spike mutation. This mutation is known to enhance the virus's infectivity and ability to form syncytia, and also makes it more resistant to neutralising antibodies, especially when combined with other SARS-CoV-2 VOCs [140]. Interestingly, while this mutation has been observed in other VOCs, such as the Alpha variant [140], we didn’t find it in any other sequences from Republic of Moldova except the Eta. Spike P681H mutation emerged early in 2021 in the Republic of Moldova and was found in 531 isolates.

One variant found in Moldovan population in September 2020 carry H49Y amino acid change in the Spike protein. H49Y mutation was described in a cluster from Mexico in early 2020 [53].

The P681H mutation is a substitution of the amino acid proline (P) to histidine (H) at position 681 in the Spike protein of SARS-CoV-2. This mutation is located in the furin cleavage site of the Spike protein, which is important for the virus to enter and infect host cells. The fact that the P681H mutation was found in 351 out of 542 samples (from February 2021 to January 2022) suggests that this variant may have been circulating widely during that time period. The P681H mutation has been associated with increased transmissibility and potentially with immune escape [141]. However, its exact impact on the severity of the disease and the effectiveness of vaccines and treatments is still under investigation.

It is important to monitor the prevalence of this and other mutations in the Spike protein, as they may have implications for the effectiveness of vaccines and treatments, and for the overall control of the pandemic. It is also crucial to continue following public health guidelines and

vaccination recommendations to reduce the spread of SARS-CoV-2 and mitigate the impact of the pandemic.

Nucleocapsid mutation G204R contribute to the increased transmission and virulence [143]. The G204R mutation was detected in 358 isolates from the Republic of Moldova from which 11 (3%) were circulating in 2020, 45 (12.5%) in 2021 and 302 (84.5%) in 2022.

Mutations are also observed in NSP2, NSP3, NSP4, NSP5, NSP6, NSP8, NSP9, NSP10, NSP12, NSP13, NSP14, NSP15, NSP16, and the spike protein, that play a significant role in infectious capability and differentiation mechanism of SARS-CoV-2. Amino acid substitutions were also observed in: NS3, NS8, NS7a, NS7b, N, E and M. In all samples was noted the Spike mutation D614G that emerged in early summer 2020 and became common in many European countries in autumn 2020.

The results suggest that SARS-CoV-2 is evolving rapidly in Moldova, with multiple mutations arising in the circulating strains. The detection of several mutations associated with increased transmissibility, virulence, and vaccine resistance is concerning and highlights the need for continued monitoring and surveillance of SARS-CoV-2 evolution in the Republic of Moldova. The comparison of the 19 sequences obtained in this study with those available in GISAID provides important insights into the diversity and evolution of SARS-CoV-2 in Moldova and its relationship with other European countries.

It's worth mentioning that we made a conscious effort to ensure that the genetic sequences collected in this study were made available to the public as quickly as possible. On average, it only took 68 days from the time the samples were collected to when they were submitted to GISAID for analysis [10].

The main focus of this study was to provide insights into the molecular evolution of the SARS-CoV-2 virus within Republic of Moldova, as well as within Europe as a whole. By framing the genetic data collected within this context, we were able to identify patterns and trends that shed light on how the virus is changing over time and across different regions.

Overall, this research represents an important contribution to our understanding of SARS-CoV-2 and how it is evolving, which is crucial for developing effective public health strategies to contain the spread of the virus and mitigate its impact on society. Furthermore, it highlights the need for increased investment in sequencing facilities and expertise in low-resource countries to improve their capacity for real-time genomic surveillance of emerging infectious diseases.

In conclusion, the study conducted at Nicolae Testemitanu State University and Pharmacy in the Republic of Moldova, in collaboration with Alfa Diagnostica Laboratory, demonstrates the importance of early and accurate detection of SARS-CoV-2. The use of advanced laboratory techniques and collaborations with leading research institutions, such as the ICGEB, can help

further our understanding of the virus and facilitate the development of effective treatments and vaccines.

3.2.2. Discussions

The results of this study provide valuable insights into the molecular evolution of SARS-CoV-2 in the Republic of Moldova. Through genomic sequencing, we identified multiple amino acid substitutions in the spike protein and non-structural proteins of the virus, including the highly transmissible Delta and Omicron variants. Our findings suggest that the demographic and geographical features of the country, including the high emigration rate of Moldovans to neighboring countries [143] and the low number of visitors to the country [144] have contributed to the unique molecular diversity and adaptation of the virus within the local population, potentially contributing to changes in viral fitness and pathogenicity.

Comparison of the 19 sequences obtained in this study with a larger dataset of 505 nucleotide sequences from several European countries revealed a high degree of genetic diversity among circulating SARS-CoV-2 strains. However, phylogenetic analysis indicated that the Moldovan isolates were closely related to other European isolates, suggesting ongoing transmission and spread of the virus within the continent.

The discovery of the Delta variant in the Republic of Moldova in June 2021 and the prevalence of its three clades indicate that this variant has become established in the region. The dominance of AY.122 in Russia, AY.43 in Romania and Italy, and AY.4.2.3 in Moldova and its neighboring counties, highlights the need for close monitoring of the distribution and prevalence of different lineages of the virus [145]. The investigation conducted in this study has identified the presence of the three Delta clades, namely 21J (Delta) with eight lineages, 21I (Delta) lineage AY.9, and clade 21A (Delta) lineage AY.54 between March 2020 to May 2021. The AY.122 variant was found to be predominant in Russia[146], whereas AY.43 variant was found to be predominant in Romania[145] and Italy[147]. The prevalence of AY.4.2.3 was observed to be 12% in Moldova, whereas in other countries, it constituted a negligible proportion of 1%. Over 50% of these sequences were acquired from patients residing in the Iasi and Vaslui counties that share a border with Moldova [145].

Furthermore, our study identified the presence of BA.2 and BA.2.9 variants in Moldova, with BA.2.9 becoming dominant in March 2021. It is worth noticing that the BA.2 was found in Poland, too [148]. The emergence of the BA.2.9 variant and its rapid dominance over the previously dominant BA.2 variant in Moldova and Poland is a significant finding of this study. Despite only one amino acid (ORF3a: H78Y) change and two nucleotide (C22792T, C25624T) modifications differentiating BA.2.9 from BA.2, there is no evidence of increased transmissibility,

infectious duration, or immune evasion of the former. The initial proportion of BA.2 was higher than that of BA.2.9 in both Moldova and Poland in February, but the situation reversed in March with BA.2.9 becoming the dominant variant. While this could be due to the refugee crisis causing a significant change in the initial proportion of the two variants, further studies are needed to investigate the underlying factors and mechanisms responsible for the shift in dominance. Additionally, tracking the prevalence and distribution of BA.2.9 sublineage in other countries and regions may provide valuable insights into the molecular evolution of SARS-CoV-2 and the emergence of new variants.

The presence of D614G in both the Omicron and Delta variants is particularly interesting, as previous studies have shown that this substitution is associated with neurovirulence [149,150], potentially enabling the virus to enter the central nervous system through the olfactory nerve [151]. However, within the Omicron variant, the absence of the E484K mutation observed in the Delta variant is notable, with E484Q present in the same locus instead. This unique combination of mutations may contribute to the increased infectivity/transmissibility of the Omicron variant, as well as potentially reducing the efficacy of monoclonal antibodies and targeted vaccines [152]. The observed conserved amino acid substitutions, namely G142D, P681L, and D614G, exhibit noteworthy evolutionary infiltration in both variants.

Despite the valuable insights gained from this study, there are some limitations that need to be acknowledged. The sample size analysed in this study was relatively small, and the time period covered was short, which may limit the generalizability of our findings. Additionally, the technical limitations of the study, such as the exclusion of samples with low copy numbers, could have also affected our results. Finally, the study focused solely on the molecular evolution of SARS-CoV-2 in the Republic of Moldova, and further research is needed to explore the epidemiological and clinical implications of these findings.

Overall, this study provides important insights into the molecular evolution of SARS-CoV-2 in the Republic of Moldova and highlights the importance of continued genomic surveillance of SARS-CoV-2 to track the distribution and prevalence of different lineages of the virus in the region and monitor potential changes in viral fitness, transmissibility, and pathogenicity. Further research is needed to determine the functional significance of the observed amino acid substitutions and their impact on viral pathogenesis and vaccine efficacy. The findings of this study could have important implications for the development of effective control strategies to mitigate the ongoing COVID-19 pandemic.

4. HUMORAL IMMUNE RESPONSES TO SARS-COV-2 IN SINOPHARM VACCINATED AND CONVALESCENT INDIVIDUALS

Background

Given the absence of any authorised pharmaceutical or immunological intervention for COVID-19 [154], the immune system represents the most effective means of protection. The immune reaction to SARS-CoV-2 encompasses both cellular immunity and the generation of antibodies [155]. The clearance and elimination of virus-infected cells is a crucial function carried out by cytotoxic T cells [155].

The production of neutralising antibodies constitutes a crucial defensive mechanism in combating the novel SARS-CoV-2 coronavirus. The antibodies possess the ability to eradicate viral particles and exhibit promising prospects for employment in the prophylaxis and therapy of SARS-CoV-2 infection.

The SARS-CoV-2 virus's S-glycoprotein, also known as the spike protein, facilitates host cell penetration and is the primary focus of neutralising antibodies. The identification of antibodies against SARS-CoV-2 does not provide conclusive evidence of safeguarding immunity, and the factors that ensure protection against COVID-19 have yet to be determined [156,157].

The identification of individuals who have been previously infected with SARS-CoV-2 is crucial for the healthcare system. Serological tests for COVID-19 can serve this purpose, as opposed to polymerase chain reaction (PCR) and other rapid diagnostic tests that only detect the presence of viral material in infected individuals at the time of testing [158].

4.1. Materials and methods

4.1.1. Sample size

A recent study which examined the effectiveness of the Sinopharm COVID-19 vaccine found that in order to accurately identify individuals who have developed positive SARS-CoV-2 antibodies after receiving the second dose of the vaccine, a minimum of 78 participants had to be included in the study. This conclusion was reached by utilizing the One Proportion Power Analysis method within the NCSS & PASS Program to ensure that it had a high level of statistical power at 80%, as well as a target level of significance of 5%. By doing so, the research was able to provide more reliable and robust findings regarding the efficacy of the Sinopharm vaccine [159].

4.1.2. Specimens' collection

Blood samples were collected from both individuals who have recovered from COVID-19 and healthy donors at the National Transfusion Centre in the Republic of Moldova. Specifically,

plasma samples were taken from 100 individuals between the ages of 40 and 60 who had tested positive for SARS-CoV-2 during their initial diagnosis but were negative at 14 days after clinical recovery. These samples were collected between September and October of 2020.

Fully vaccinated individuals were also recruited from Nicolae Testemitanu State University of Medicine and Pharmacy. In order to be classified as fully vaccinated, individuals had to have received a second dose of the BBIBP-CorV vaccine at least 14 days before sample collection. Sera were drawn, at ALFA Diagnostica Laboratory, from 100 vaccinated people. with a female-to-male ratio of 68% to 32%. The mean age was 23 (95% CI: 22-23.9), with a median age of 22 (95% CI: 22-23). The age range varied from 19 to 49, with a standard deviation of 5.08. Among these vaccinated individuals, 26 participants with a female-to-male ratio of 81% to 19% had contracted COVID-19 before receiving the vaccine. The mean age was 22.8 (95% CI: 21.5-23.8), with a median age of 22 (95% CI: 20-22.5). The age range varied from 19 to 33, with a standard deviation of 2.97. These samples were collected between May and June of 2021.

As a negative control, 96 samples from healthy adult bank donors in 2018 were included in the analysis.

All samples were analysed in accordance with Protocol No 3/24.01.22, which the Research Ethics Committee of Nicolae Testemitanu State University of Medicine and Pharmacy approved. All experiments complied with relevant guidelines and regulations, and written informed consent was obtained from all participants. Before the study, all patient data were anonymized.

Blood sample management

The blood samples were collected in Heparin and serum-gel monovette tubes, produced by Greiner Bio-One. After the blood was drawn, centrifugation was used to separate the heparinized plasma and serum samples from the rest of the blood components. The samples were then aliquoted and treated for an hour at 56°C to inactivate complement components and SARS-CoV-2 virions. Finally, the samples were stored at -80°C until they were ready for analysis. This ensured that the samples remained stable and free from any contamination that could have affected the results of the analysis.

4.1.3. Cell lines

Human Embryonic Kidney 293 cells abbreviated to HEK 293 (ATCC, Rockville, MD, USA, CRL-1573) are an immortalized cell line [160] HEK 293T (ATCC, Rockville, MD, USA, CRL-11268) is a HEK 293 derivative human cell line that expresses a mutant version of the SV40 large T antigen. The transfection used to create 293T (involving plasmid pRSV-1609) conferred neomycin/G418 resistance and expression of the tsA1609 allele of SV40 large T antigen. Due to

the expression of SV40 large T antigen, transfected plasmid DNAs that carry the SV40 origin of replication can replicate in 293T and will transiently maintain a high copy number; this can greatly increase the amount of recombinant protein expression or retrovirus that can be produced from the cells [161] HEK293T (ATCC, Rockville, MD, USA, CRL-3216) were grown in Dulbecco's Modified Eagle's Medium (DMEM, with D-glucose (1g/L) and pyruvate (GIBCO Ref. 21885-025), supplemented with 10% decomplemented Fetal Bovine Serum (FBS) (GIBCO Ref. 10270-106) and 50µg/mL Gentamicin, and incubated at 37°C, 5% CO2, 80% humidity.

Huh7-hACE2 and HEK293-ACE2 are stable cell lines that express on its membrane human ACE2 receptor. As described before, these cells were generated by transduction of a lentivector expressing human ACE2 [162]. The Huh7-hACE2 and HEK293-ACE2 cells were cultured in complete DMEM medium supplemented with Puromycin 1µg/mL (Invitrogen) and kept in an incubator at 37°C and 5% of CO2. To confirm the expression level of ACE2 on the HEK293-ACE2 cell line, immunostaining and Flow Cytometry were used. To do this, 5×10^5 cells were incubated with blocking solution (PBS - 3% BSA - 0,2% NaN3) for 40 minutes at 4 °C, and then incubated with primary antibody Polyclonal Goat IgG anti-ACE2 (diluted 1:50 in blocking solution, antibody Catalog Number AF933; Biotechne) and secondary antibody Fluorescein (FITC)-conjugated AffiniPure Rabbit anti-Goat IgG, FC γ-specific (diluted 1:500 in blocking solution, Code Number 305-095-008; Jackson Immuno Research) for 1 hour at 4°C each in two different reactions. Following washing with PBS – 0,2% NaN3, cells were resuspended in PBS – 10mM EDTA pH 8 and the cytofluorimetry data were analysed using FlowJo software V10 (BD) [17].

4.1.4. Flow cytometer: BD Accuri ™ C6 plus

The BD Accuri™ C6 Plus (figure 15) is a flow cytometer that is used in many fields of biological and medical research. The instrument uses laser-based technology to analyze and sort cells based on their physical and chemical properties. The instrument has a wide range of applications, from basic research to clinical diagnostics. It can be used to analyze cell populations in a variety of sample types, including blood, tissue, and cell cultures. Researchers can use it to identify and quantify different cell types, study cell signaling pathways, and investigate disease mechanisms. The BD Accuri™ C6 Plus has a high throughput capacity, with a sample acquisition rate of up to 10,000 cells per second. It also has a large dynamic range, allowing for the detection of rare cells or events within a population. The instrument is compatible with a range of sample preparation methods, including cell staining and labeling, and can be used in conjunction with other techniques such as PCR and Western blotting. It utilizes a laser to excite fluorescent dyes and other markers on the cells or particles passing through the instrument's flow cell. The resulting

signals are detected by photomultiplier tubes and processed by the instrument's software to provide data on the size, shape, and other characteristics of the cells or particles. The BD Accuri ™ C6 Plus is equipped with a blue and red laser, two light scatter detectors, and four fluorescence detectors with optical filters optimized for detecting popular fluorochromes, such as FITC, PE, PerCP-Cy™5.5, and APC, as well as newer polymer dyes like BD Horizon Brilliant™ Blue 515. In addition, it can analyze different types of fluorescent proteins, -including GFP, YFP, and mCherry.

Figure 15. **BD Accuri™ C6 Plus flow cytometer**

Flow cytometry is a powerful tool in biomedical research and clinical diagnostics because it allows researchers and clinicians to analyze large numbers of cells or particles rapidly and with high accuracy. It is commonly used in immunology, hematology, microbiology, and oncology, among other fields.

4.1.5. Synthesis of plasmid DNA carrying SARS-CoV-2 Omicron Spike glycoprotein sequence

The plasmid vector, pcDNA 3.1 (+), selected for the cloning of the Spike Omicron gene was first linearized. The DNA was digested with the following restriction enzyme, EcoRI-HF® (NEB) and XhoI (NEB), and digestion was run on 1% agarose gel TAE 1X -Ethiudium Bromide 0,5µg/mL (TAE 1X, 0,04 M Tris-Acetate, 0,001 M EDTA pH 8). After the cutting of the linearized DNA from the gel, the DNA extraction was then carried out using a NucleoSpin® Gel and PCR Clean-up kit, following the manufacture's protocol. The recovered DNA was quantified by Nanodropand and used together with 4 DNA fragments of Spike for the setting of Gibson Assembly® Reaction Cloning Kit (NEB). Gibson Assembly® Kit is a robust exonuclease-based method to assemble DNA seamlessly and in the correct order. The nucleotide sequence of the Omicron Spike was obtained from the original strain Wuhan by online database (GenBank NCBI reference sequence: NC_045512.2), according to the omicron's mutations the sequence was codon

optimized for the expression in mammalian cells and we changed the corresponding codons. Spike sequence gene was divided into 4 fragments and at the 3'- 5' ends of each fragment 30-35 homologous nucleotides were added in order to pair with the right fragments and in the right order (figure 16). The four fragments (or inserts) were obtained as synthetic dsDNA blocks by IDT company (Integrated DNA Technologies, Inc). The reaction containing the 4 fragments, the linearized vector (pcDNA3.1 (+)) and the *Gibson Assembly Master Mix 2X*, in the ratio reported in table 2, was incubated at 50 ° C for 60 minutes.

Table 2. **DNA amounts in gibson assembly reaction components**

	µL	pmol of DNA
Gibson Assembly Master Mix (2X)	10	/
Vector pcDNA3.1 (+)	2.8	0.0028
Insert A	0.9	0.084
Insert B	2.8	0.084
Insert C	2.9	0.084
Insert D	0.7	0.084

Following incubation, the samples were stored on ice or at –20 ° C, and 2 µL of this reaction were used to transform NEB 5-alpha Competent *E.coli* cells (DH5α, provided with the kit).

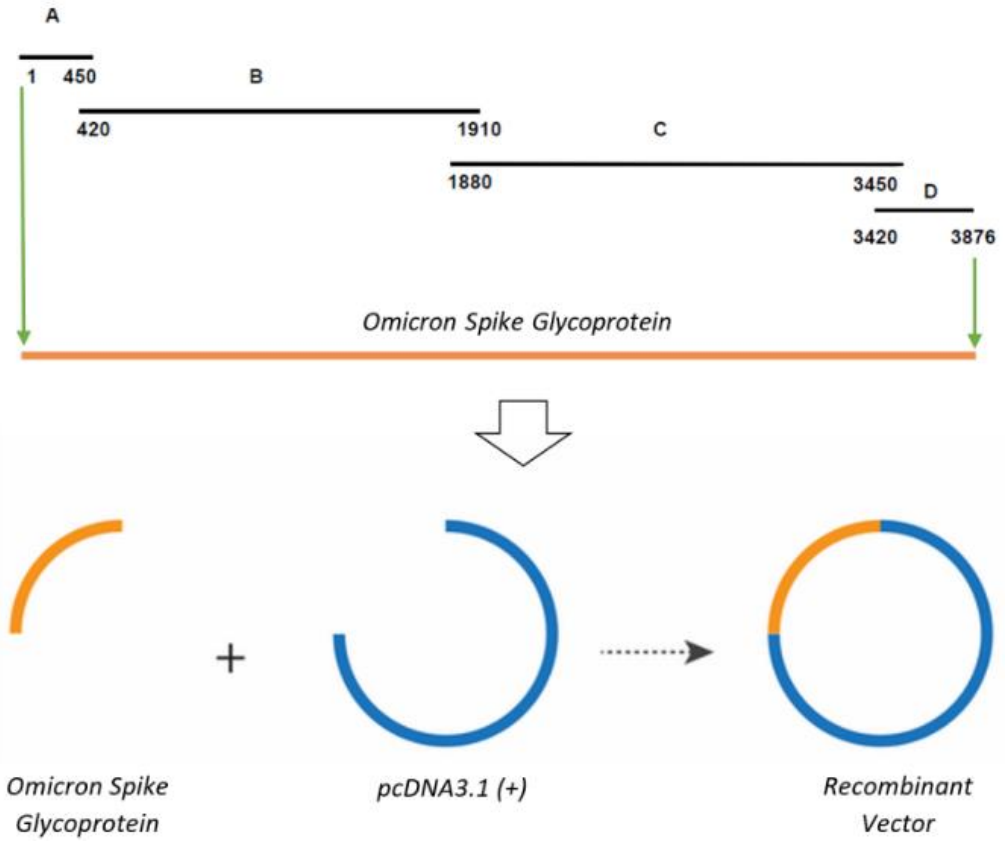


Figure 16. **Overview of the gibson assembly cloning method**

4.1.6. Bacterial transformation and DNA extraction

Bacterial transformation is a process in which bacteria take in foreign genetic material, typically in the form of DNA. To increase the amount of plasmid DNA, bacterial transformations were carried out using DH5α Competent cells, which are *E. coli* cells that have been modified to enhance the efficiency of transformation. Two plasmids, pcDNA3 carrying D614G-Δ19 Spike sequence (gift from Prof. Massimo Pizzato, University of Trento) and pcDNA3.1 (+) carrying the Omicron Spike sequence, were transformed into these cells. DH5α cells have three mutations (recA1, endA1, and lacZΔM15) that facilitate plasmid insertion and enable blue-white screening [164].

4.1.6.1. Transformation protocol

For the transformation reaction, DH5α cells and the plasmid DNA were mixed together in a 1.5 mL Eppendorf tube. The amount of DNA used in transformation was around 10-30 ng. The tube was then placed on ice for 20/30 minutes, and after this incubation, a heat shock of the bacteria was done, using thermomixer, at 42°C for 30 seconds. Following an incubation on ice for 5 minutes, LB medium (Luria-Bertani) was added, and the reaction was incubated at 37 °C for 30-60 minutes. The transformation reactions were plated on LB agar plates containing ampicillin (AMP 75 µg/mL), and the plates were left overnight at 37 °C. The following day a colony was selected and picked, then grown in 15 mL tubes containing LB medium supplemented with AMP (AMP 100 µg/mL) and left in incubation overnight at 37°C in gently shaking.

4.1.6.2. Plasmid DNA isolation with NucleoSpin Kit

Miniprep DNA extractions were performed using the Nucelospin® Plasmid kit (Macherey-Nagel). Briefly, following the kit instruction and using the buffers provided with the kit, bacterial cells were lysed, and the lysate clarified by centrifugation. A NucleoSpin® Plasmid / Plasmid (NoLid) Column was placed in a Collection Tube (2 mL) and the supernatant from the prior centrifugation was filtered by centrifuge. After 2 wash step and a further centrifuge to dry the membrane, the plasmid DNA was eluted in water and quantified by Nanodrop.

4.1.6.3. Restriction enzymatic digestions of plasmid DNA coding for Omicron Spike Glycoprotein

The DNA eluted from the miniprep column kit was digested with 3 different restriction enzymes in order to select positive clones for sequencing. Two different reactions were carried out:

1) HindIII/XbaI
2) EcoRI

The digestion reactions were prepared, following NEB protocol, each with the proper DNA, enzyme, and buffer solution, and left in incubation at 37°C for 1-2 hours. The digestions were run in 1% agarose in TBE 1X (0,089 M Tris-Borate, 0,089 M Boric Acid, 0,02M EDTA pH8) and Ethidium Bromide.

4.1.6.4. Sequencing of DNA coding for Omicron Spike Glycoprotein

To verify if the nucleotide Omicron spike sequence was correctly assembled with the Gibson Assembly technique the plasmids were sent to sequencing to the Eurofins Scientific company (Sequencing Lab Ebersberg). Five primers were chosen in order to sequence the full length:

1) pcDNA3_forward (GGC TAA CTA GAG AAC CCA CTG)
2) pcDNA3_reverse (GGC AAC TAG AAG GCA CAG TC)
3) 1821 Forward (CGA TCC TCT TAG TGA GAC TAA GTG)
4) 2727 Forward (AAC CGT TCG AAC GCG AC)
5) 4310 Reverse (CTG CTT CTA CCT TGT CG).

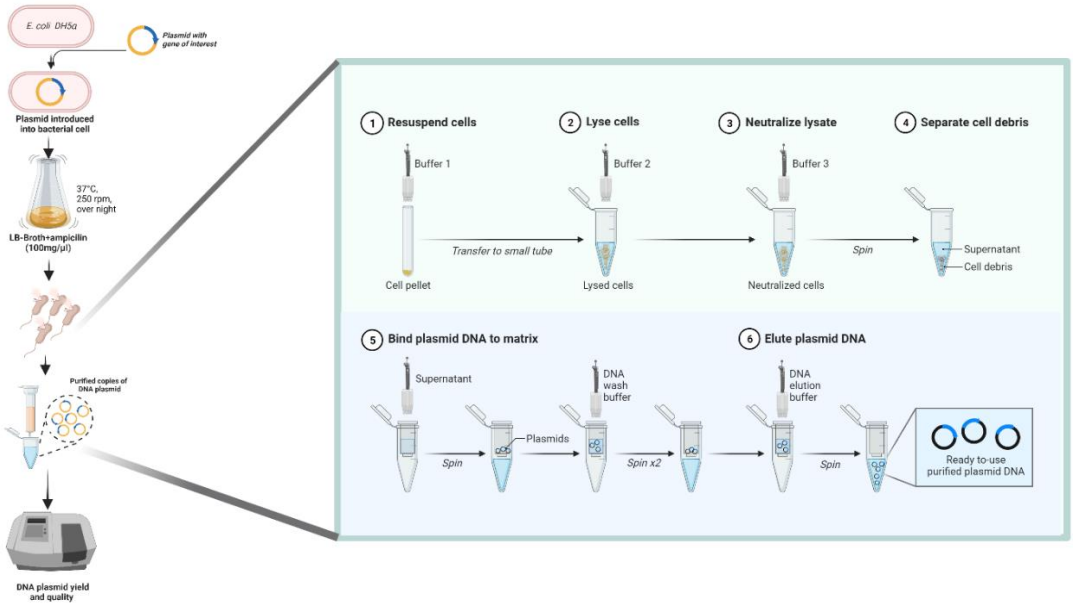


Figure 17. **Amplification of DNA fragments for integration into a plasmid vector**

4.1.7. Plasmid DNA preparation for molecular cloning

Once the correct sequence was verified by sequencing, the corresponding plasmid DNA was selected, transformed in *E. coli* DH5α and left to grow in an Erlenmeyer flask with LB-Broth and ampicillin (100mg/µL) at 37°C and with constant shaking (250 rpm) overnight. The plasmid DNA was extracted from transformed cells with the Midiprep NucleoBond® Xtra Midi Endotoxin-Free kit (Macherey-Nagel), following the manufacture's protocol. In brief, the bacterial

lysate was placed in the nucleobond column, which is an anionic exchange-based column. Eluted DNA was precipitate with isopropanol and resuspended in TE or water. The plasmid DNA quantity and quality was then determined by UV spectroscop (figure 17).

4.1.8. Lentivirus pseudotyping using SARS-CoV-2 Spike protein

To produce SARS-CoV-2 Spike pseudoviruses that express Green Fluorescent Protein (GFP) protein, a lentivector system was utilized [164,165] This involved the transfection of three plasmids, including pLVTHM which codes for a GFP reporter, the packaging plasmid psPAX2, and pCDNA3 which carries the D614G SPIKEΔCyto, into HEK-293T cells (figure 20a).

Enhancing transfection efficiency to optimize the production of pseudotyped lentivirus carrying SARS-CoV-2 Spike protein

A day before (*Day 0*) transfection, HEK293T cells were re-suspended in the same medium in which they grew and centrifuged for 5 min, 1000 rpm, room temperature. Then the supernatant was discarded, and the sediment was resuspended with DMEM medium containing 10% FBS. $3x10^6$ cells were seeded onto a Ø10cm TC-dish and grown to 70% - 80% confluence, overnight, at 37°C, 5% CO2, 80% relative humidity. Figure 18 depicts a representation of how the cells are expected to appear.

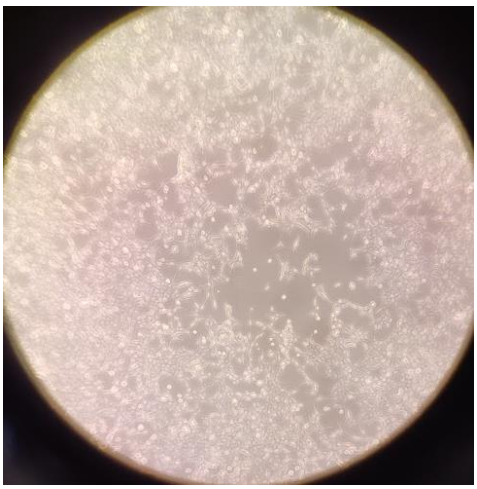


Figure 18. **Example of the confluency expected prior to transfection of HEK293T cells with plasmids**

The following day *(Day 1*), 3 - 4 hours before transfection the medium was removed and replaced with fresh medium. To generate Lentivirus Pseudotyped with SARS-CoV-2 spike protein, cells were transfected with the 3 DNA mixes assembled following calcium phosphate transfection method [112]. Briefly, for each transfection reaction, 3 sets of sterile 1.5 mL tubes were prepared.

In one tube 500 µL of Hepes Buffer Saline (HBS, 280 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4, 12 mM D-glucosio, 50mM HEPES) was added and in the second tube the 3 DNA plus TE 0.1X were added in a final volume of 100 µL. The 3 DNA mixes were assembled as reported in table 3.

The third tube, which contained 338 µL of water, was used to mix 10 µL of 2M CaCl2, the DNA mixture from table 3, and 52 µL of 2M CaCl2. The addition of CaCl2 resulted in the precipitation of DNA. Then 2 ml sterile pipette connected to a Pipet-Aid was immersed in the Eppendorf tube containing 500 µL of HBS2X and air was pushed off in order to produce bubbles. At the same time the DNA mixture (water-CaCl2-DNAs-CaCl2) was added drop by drop to the HBS keeping bubbles production, at least 1 minute more. 1 ml solution, containing DNA crystals was added in a dropwise fashion to the cells with gentle swirling [166]. After overnight incubation at 37°C, 5% CO2, 80% relative humidity, the transfection medium was removed, cells were washed with 5 mL DMEM-serum free or PBS and supplemented with 10 mL fresh medium containing 2% FBS, then they were incubated 48 hours at 37°C, 5% CO2, 80% relative humidity. Forty-eight hours' post-transfection the GFP expression was monitored using the Leica Fluorescent microscope (figure 19).

Table 3. **Molar ratios of DNA plasmids used in transfection reaction to produce the three lentiviruses**

1st transfection reaction mix: *SARS-CoV-2 D614G variant*	**2nd transfection reaction mix: *SARS-CoV-2 Omicron variant***	**3rd transfection reaction mix: *VSV Lentivirus***	**Molar ratio**
psPAX2	*psPAX2*	*psPAX2*	*1.5*
***pcDNA3*-Spike**	*pcDNA3.1 (+)*-Omicron	*pMD2.G*	*1*
plVTHM	*plVTHM*	*plVTHM*	*3*
Final Volume (DNAs + TE0.1X) = 100 µL			

Note: *psPAX2 (lentiviral packaging plasmid); plVTHM (eGFP, reporter gene); pcDNA3/3.1 (+) (Spike glycoprotein); pMD2.G (VSV envelope)*

Lentiviruses undergo the lysogenic cycle, which results in the release of virus into the medium without cell lysis. Therefore, the supernatant containing the lentivirus pseudotyped with SARS-CoV-2 spike protein was gently aspirated at 5th day post-transfection, and the cells were discarded. To remove any cell debris and multi vesicular bodies (MVB), the supernatant was filtered through a 0.45 µM Millex-HV Syringe Filter. The lentiviruses encoding GFP were then aliquoted and cryopreserved at -80°C until use. The schematic abstract of the SARS-CoV-2 pseudotyped lentivirus is shown in figure 20a.

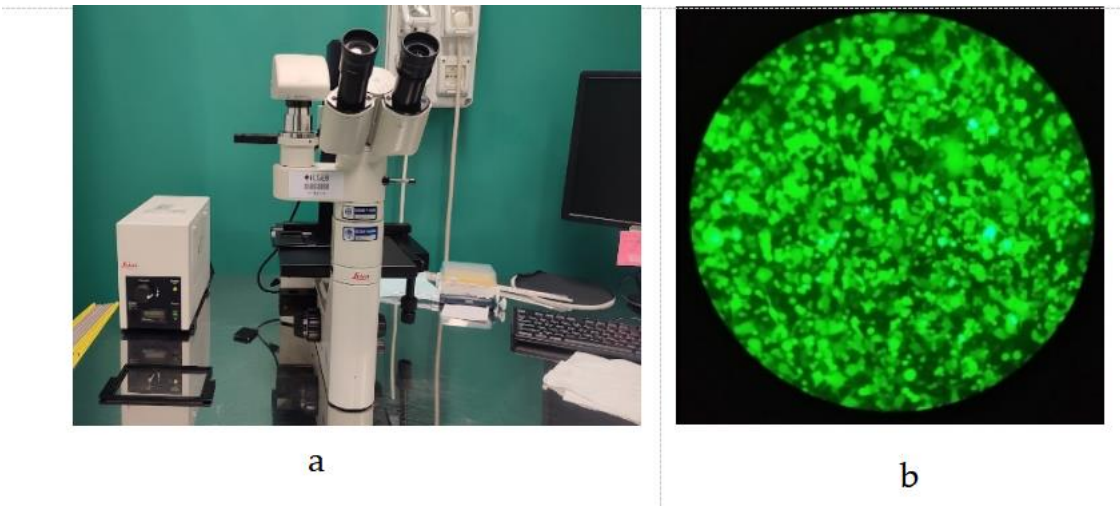


Figure 19. **Visualization of protein expression in HEK293T cells**
a) Fluorescent Microscope Leica b) Green Fluorescent Protein (GFP) expression in HEK293T cells

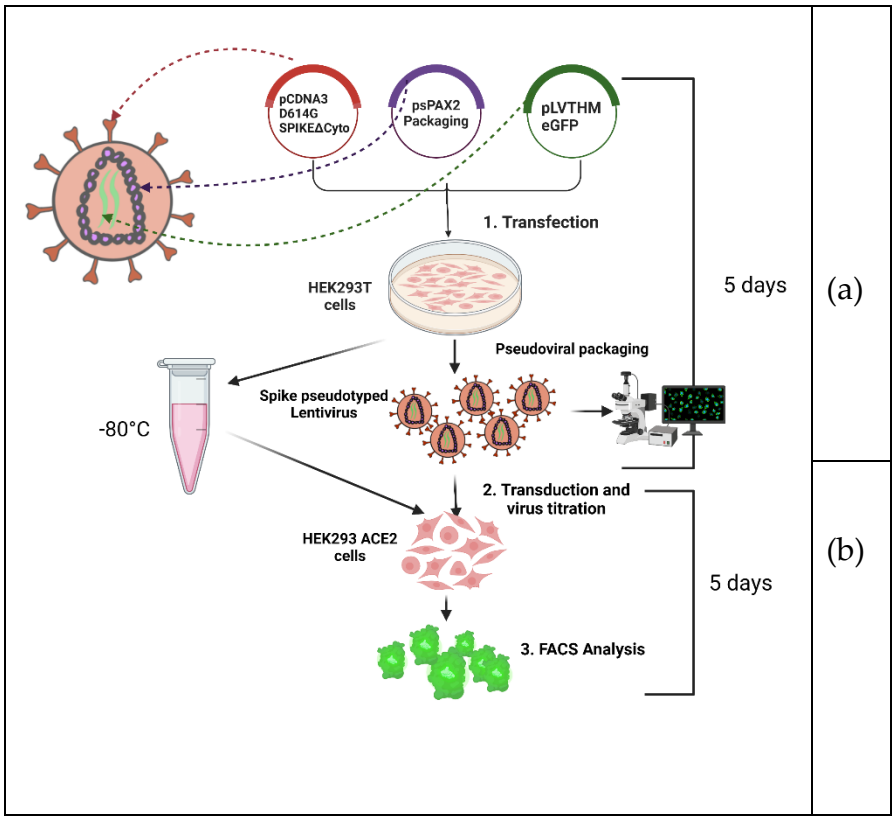


Figure 20. **A schematic of SARS-CoV-2 Pseudotyped Lentivirus System**
(a) Production of lentivirus particles pseudotyped with SARS-CoV-2 Spike (S) protein
(b) Transduction and titration of Pseudotyped Lentiviral Particles with Spike SARS-CoV-2

4.1.9. Transduction and titration of pseudotyped lentiviral particles with Spike SARS-CoV-2

To determine the lentiviruses concentration, various amounts of the viral suspension were used to infect HEK293-ACE2 cells which express GFP upon infection. The resulting GFP expression was measured using flow cytometry as shown in figure 20b.

Initially, $3x10^4$ HEK293-ACE2 cells/well were seeded onto a 96-well plate (FALCON, Ref#353072) and some extra wells were seeded to count cells at the moment of transduction, then allowed to incubate overnight at 37°C, 5% CO2, 98% relative humidity. The next day, control cells were collected and counted, while lentivirus encoding GFP was thawed and diluted in 2% FBS DMEM. The cells were transduced with different dilutions of the virus, ranging from 150 µL to 1.5 µL, along with positive and negative controls. As positive control undiluted virus was used. As negative control for transfection was used medium free of virus. The experiment was performed in duplicate. After 72 h incubation the supernatant was removed and cells were collected and resuspended in 5mM EDTA pH8, then analysed by flow cytometry (BD Accuri C6). The percentage of infected cells, cell count, and the dilution of virus preparation were used to calculate the lentiviral titre, expressed as transduced units per milliliter (TU/mL), using a specific formula:

$$\textbf{Titre (TU/ml)} = (N \times P)/(V \times D) \quad (1)$$

(N = Cell Number in each well used for infection on Day 1; P = percentage of GFP positive cells; V = virus volume used for infection in each well; the V (ml) = µl×10-3; D = dilution fold; TU = transduction unit)

4.1.10. Assessment of SARS-CoV-2 neutralisation using a lentivirus pseudovirus system

The titrated lentivirus (D614G-Δ19 variant, not tested with Omicron variant) was used for the neutralisation assays, which were conducted using either a Flow Cytometry or a High Content screening system.

Assessment of SARS-CoV-2 neutralisation with flow cytometry: a step-by-step protocol

As shown in figure 21, HEK293-ACE2 cells were seeded in a 96-well culture plate (SAESTEDT, Ref#83.3924) at a density of $3x10^5$ cells per well left to grow overnight at 37°C, 5% CO2. Next day, duplicate samples of serum or CP were prepared in DMEM-2% FBS at three different concentrations using fivefold serial dilutions. These samples were mixed with an equal amount of pseudovirus preparation and incubated for 1.5 hours at 37 ◦C and 5% CO2, resulting in a starting dilution of 1:10. Afterward, the cell plate's supernatant was replaced with 200 µL of

virus-antibody mixtures, and incubated for 72 hours at 37°C, + 5%CO2, 80% humidity. The plate included negative pre-COVID donor serum (dilution 1:12.5), virus alone and not transduced cells as controls for the neutralisation assay.

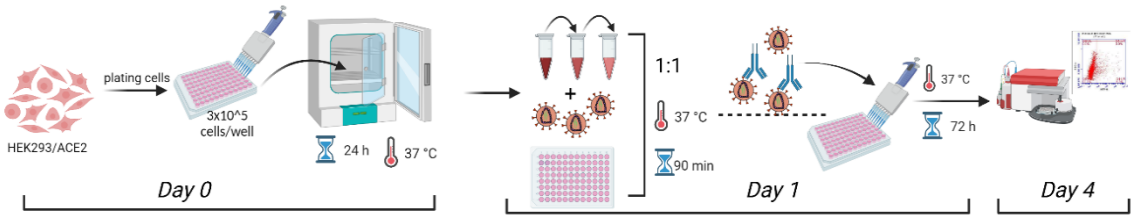
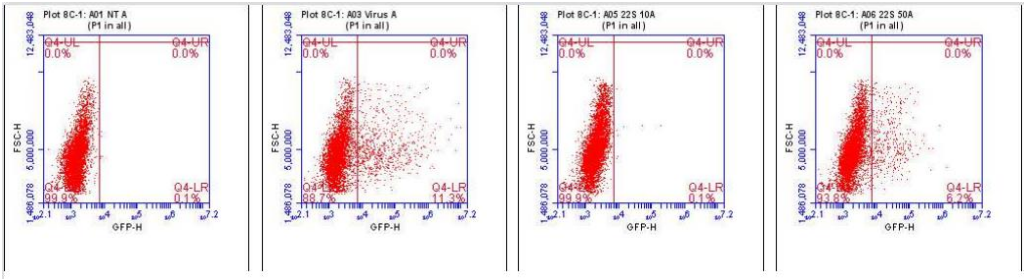


Figure 21. **Schematic abstract of SARS-CoV-2/lentivirus neutralisation assay using flow-cytometry**

Following the incubation period, cells were collected, resuspended in phosphate-buffered saline (PBS) 5mM EDTA and analysed for the percentage of transduced cells using FC (figure 22).



Plot 8C-1: A01 NT A Gated on (P1 in all)	Count	Volume (µL)	% of This Plot	% of All	Mean GFP-H	Mean FSC-H	CV GFP-H	CV FSC-H	Median GFP-H	Median FSC-H
This Plot	6,805	46	100.00%	68.05%	1,519.84	4,497,380.35	48.56%	27.99%		
Q4-UL	0	46	0.00%	0.00%	0.00	0.00	0.00%	0.00%		
Q4-UR	0	46	0.00%	0.00%	0.00	0.00	0.00%	0.00%		
Q4-LL	6,799	46	99.91%	67.99%	1,514.33	4,496,366.95	47.18%	27.98%		
Q4-LR	6	46	0.09%	0.06%	7,758.00	5,645,738.17	7.27%	24.84%		

Plot 8C-1: A03 Virus A Gated on (P1 in all)	Count	Volume (µL)	% of This Plot	% of All	Mean GFP-H	Mean FSC-H	CV GFP-H	CV FSC-H	Median GFP-H	Median FSC-H
This Plot	6,646	53	100.00%	66.46%	23,588.55	4,656,942.68	1,128.61%	27.65%		
Q4-UL	0	53	0.00%	0.00%	0.00	0.00	0.00%	0.00%		
Q4-UR	0	53	0.00%	0.00%	0.00	0.00	0.00%	0.00%		
Q4-LL	5,897	53	88.73%	58.97%	1,881.32	4,591,088.67	52.89%	27.28%		
Q4-LR	749	53	11.27%	7.49%	194,493.14	5,175,422.11	396.92%	27.74%		

Plot 8C-1: A05 22S 10A	Count	Volume (µL)	% of This Plot	% of All	Mean GFP-H	Mean FSC-H	CV GFP-H	CV FSC-H	Median GFP-H	Median FSC-H

Figure 22. **Assessment of lentivirus transduction efficiency using BD Accuri 6 Plus Flow Cytometry**

The percentage of infection reduction and neutralisation titre were then calculated using the resulting data and the given equation [17]:

Percentage of infection reduction = 100 x (1- ($GFP_{diluted\ test\ sample}$-$GFP_{no\text{-}virus\ control}$/$GFP_{no\text{-}antibody\ control}$-$GFP_{no\text{-}virus\ control}$)) (2)

The reciprocal of the dilution at which a 50% reduction in infection is achieved was used to indicate the neutralisation titre.

High-throughput analysis of SARS-CoV-2/lentivirus neutralisation with high content imaging

We developed a high content imaging-based protocol for measuring neutralising activity against SARS-CoV-2/lentivirus pseudovirus, as shown in figure 24. To conduct the experiment, Huh7- hACE2 cells were seeded onto a PerkinElmer 96 well plate and with 4×10^3 cells in each well, and then left to incubate overnight at a temperature of 37°C and a CO_2 concentration of 5%. The next day, pseudovirus was mixed with serum samples that were fivefold serially diluted, with each well containing 100 µL of each component. Samples were tested in duplicate at three different dilutions (1:10, 1:50 and 1:250). The mixtures were incubated at 37°C with 5% CO2 for 90 minutes and then added to each well for spin infection. The positive control consisted of pseudovirus-only wells, while negative controls included media-only wells and sera from negative pre-COVID-19 donors (dilution 1:12.5). After 48 hours of incubation, the plates were treated with 4% Paraformaldehyde (PFA) for 20 min at room temperature and washed twice with PBS 1x. Cells were then treated with 4',6-diamidino-2-phenylindole (DAPI, dilution 1:1000) and incubated for 30 minutes at 37°C. The plates were washed twice with PBS 1x and 150 µL of PBS was added to each well. Digital images of the cells were taken using a high-content imaging system, the Operetta (PerkinElmer) (figure 23), with nine fields of each well at 20× magnification and analysed using the Columbus Image Data Storage and Analysis System (PerkinElmer) to determine the total number of cells and the number of cells that were transduced with GFP [17,167].

Figure 23. **Operetta system for high content imaging of lentivirus transduction efficiency**

The GFP-positive cell percentages were evaluated using the following formula [17]:

Number of Positive Cells/Total Number of Cells × 100 (3)

The percentage of neutralisation for various serum dilutions was determined using the formula [17]:

(1-(% of positive GFP cells of sample dilution – % positive GFP cells of negative control)/ (% of positive GFP cells of positive control - % positive GFP cells of negative control) x100. (4)

The % neutralisation=100 – % of transduction [168].The half-maximal neutralisation titre (NT50) was the serum dilution that reached 50% neutralisation using Excel nonlinear regression analysis (Excel Office 16) [17,100,169].

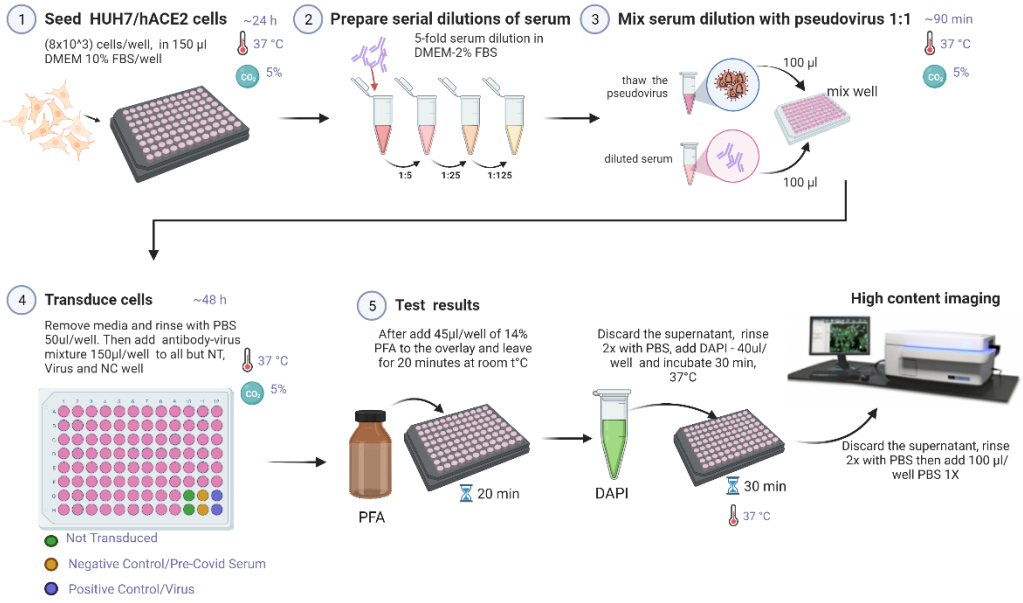


Figure 24. **SARS-CoV-2/lentivirus neutralisation assay High Content Imaging based protocol**

4.1.11. SARS-CoV-2 Spike Receptor Binding Domain direct ELISA

To test for the presence of antibodies against SARS-CoV-2, the recombinant RDB gene which had a 6x histidine tail at C-terminus was first cloned, expressed and purified using established methods [170]. As shown in figure 25, the 96-well ELISA plates were coated with 100µL of 1µg/ml purified recombinant RBD protein in PBS buffer and left to incubate overnight at 4°C. The plates were then washed with PBS-Tween 0.05% and blocked with 3% milk in PBS-Tween 0.05%, followed by adding duplicate serum or plasma samples. Two-fold serial dilutions were performed for each sample, and 100 µL of each diluted sample was added to the wells of the plates for further 2-hour incubation at room temperature. Dilutions ranged from 1:50 to 1:25600 and were performed in 1% milk in PBS-Tween 0.05%. After washing the plates three times with

PBS-Tween 0.05%, 100 µL of horseradish peroxidase (HRP)-conjugated goat anti-human IgG γ-chain (Sigma-Aldrich, Product Number: A6029) in 1% milk in PBS-Tween 0.05% (1:5000) was added to each well, then incubated 1 hour at room temperature. After a final wash, 50 µL of TMB Substrate (Sigma-Aldrich, Product Number: T444) was added for 10 minutes, and the reaction was stopped with 50 µL of 1 M H_2SO_4 solution. The optical density for each well was immediately measured using a BIORAD iMark absorbance microplate reader at 450 nm wavelength. To determine the cut-off for positivity, 100 pre-COVID sera were tested in duplicate at a dilution of 1:50, and the results were used to calculate the cut-off value based on the formula: OD Mean + 3 SD (standard deviation) [17,171].

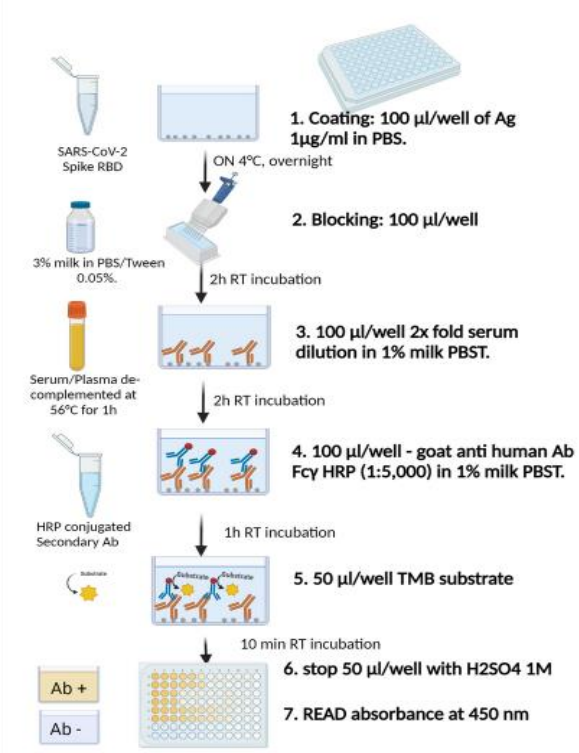


Figure 25. **ELISA protocol for anti-SARS-CoV-2 RBD IgG antibodies detection**

4.1.12. Statistical analysis

The data obtained from the study were analysed using R software version 4.2.1 from the R Foundation for Statistical Computing in Vienna, Austria [41]. Q-Q plots and the Shapiro-Wilk test were used to ensure that the data were normally distributed. The choice of parametric or non-parametric statistical tests was based on the distribution of variables.

The associations between titres of serological tests were evaluated by dividing the participants into separate groups, such as convalescent and vaccinated, and further sub-dividing the vaccinated participants into individuals who received only vaccination and those with prior infection and vaccination. Spearman's (ρ) rank correlation coefficient was used to evaluate the associations of titres obtained by ELISA, FC, and HCA. Differences in titres across different tests

and participant groups were evaluated using the Wilcoxon rank sum test, with the Wilcoxon effect size metric determining the effect size. To address multiple comparison problems, a false discovery rate (FDR) was used for multiple comparison corrections.

The statistical significance threshold was set at $p < 0.05$ in all cases.

The antibody titres in all three methods (ELISA, FC, and HCA) was calculated using Excel (Excel Office 16) nonlinear regression analysis, and the levels of antibodies were plotted using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

4.2. Results

4.2.1. Expression level of ACE2 receptor on HEK293-ACE2 cell membrane

In order to achieve efficient transduction in experiments involving SARS-CoV-2 pseudotyped lentivirus, the level of ACE2 receptor expression on the membrane of HEK 293-ACE2 was verified using immunostaining and FC analysis.

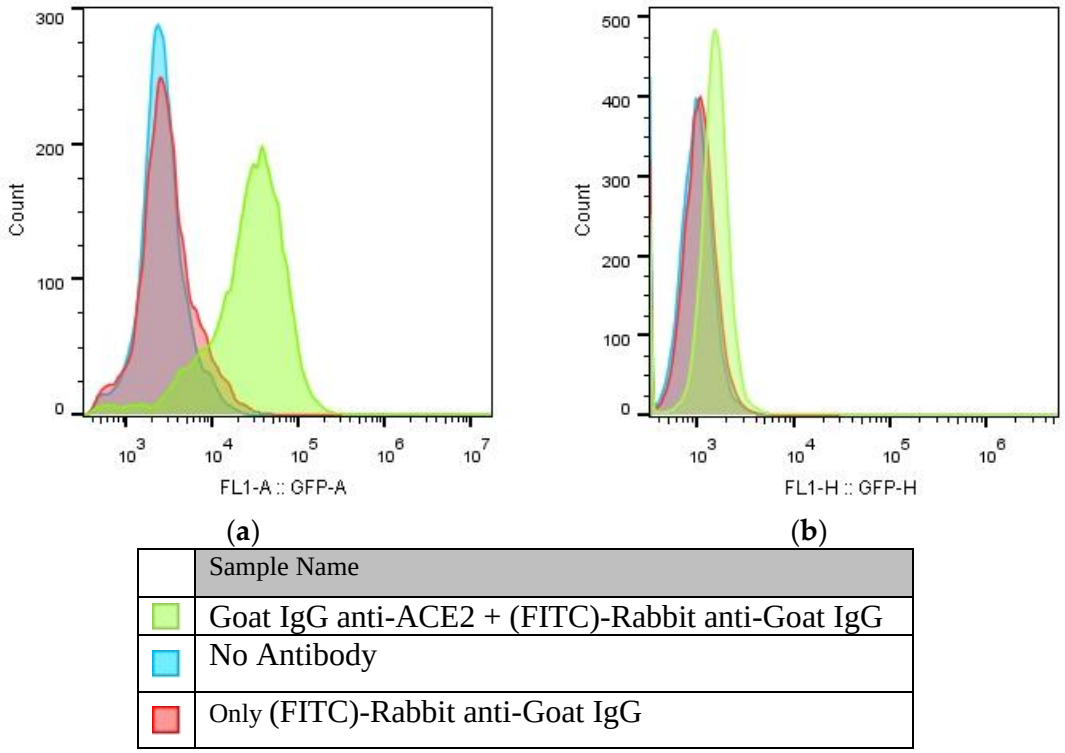


Figure 26. **The amount of ACE2 receptors present on the surface of (a) HEK 293-ACE2 cells and (b) HEK 293T cells**

A negative control was included in the experiment using wildtype HEK 293T cells incubated with the same antibodies. The results showed a positive fluorescence peak shift (figure 26, a) in HEK 293-ACE2 cells when incubated with both primary goat IgG anti-ACE2 and secondary FITC-labelled antibodies, while the control HEK 293T cells (figure 26, b) did not

exhibit any significant peak shift, which was expected. The control HEK 293T cells did not exhibit any significant peak shift, which was expected. Conversely, plot A revealed two overlapping negative peaks for the secondary antibody only (red peak) or no antibody (blue peak).

Overall, the comparison of the results obtained from the negative control and the experimental cells clearly demonstrated the assay's specificity. Thus, this experiment confirmed that the expression level of the ACE2 receptor was sufficient for subsequent experiments with SARS-CoV-2 pseudotyped lentivirus. This information is important as ACE2 receptors are responsible for allowing entry of the SARS-CoV-2 virus into cells, and studying the expression level of these receptors can help in understanding the virus's infectivity and potential treatments for COVID-19.

4.2.2. Generation of SARS-CoV-2 pseudotyped lentiviruses

The presence of GFP fluorescence was easily noticeable 72 hours after transduction, as shown in figure 27. A 2nd generation HIV-LV system was used to produce the SARS-CoV-2 Lentivirus (lentivirus particles pseudotyped with SARS-CoV-2 Spike glycoprotein). This system can be used to identify antibodies that can detect the spike protein in its natural form. To generate SARS-CoV-2 Lentivirus, we used the *plVTHM* lentiviral transfer plasmid, which encodes for the reporter gene (eGFP), the *psPAX2* HIV packaging plasmid (which encodes for gag-pol) and a plasmid carrying the sequence encoding for the structure glycoprotein Spike, *pcDNA3*-SARS-CoV-2-Spike D614GΔ19. The eGFP plasmid sequence is flanked by long terminal repeats (LTRs) that facilitate host genome integration. We conducted titration experiments to ensure the accuracy of the lentivirus SARS-CoV-2 transduction. The study used the same protocol but replaced the plasmid coding for the spike proteins with the plasmid *pMD2.G* encoding for the VSV envelope. These experiments produced a lentivirus expressing Vesicular Stomatitis Virus (VSV) envelope. The VSV lentivirus was used as a positive control since it can infect both HEK 293-ACE2 and HEK 293 cell lines, while the SARS-CoV-2 lentivirus can infect only HEK 293 expressing ACE2 receptor on the membrane [17]. We produced a SARS-CoV-2 lentivirus carrying the D614G mutation, accompanied by the deletion (Δ19) of the last 19 amino acids at the C-terminal domain, corresponding to the ER-retention motif. Previous studies have demonstrated that this variant increases the pseudotyping efficiency [172]. The deletion region is located at the cytoplasmic region and appears to remove steric interference caused by the cytoplasmic tail with the viral capsid [173]. Comparing the transduction of these two lentiviruses in HEK 293 and HEK 293-ACE2 cells, figure 27 shows the specificity of the SARS-CoV-2 lentivirus since it only infects cells expressing the ACE2 receptor on the membrane. We observed GFP expression following

Lentivirus transduction, indicating successful binding of the SARS-CoV-2 Spike-expressing Lentivirus to HEK 293-ACE2 cells.

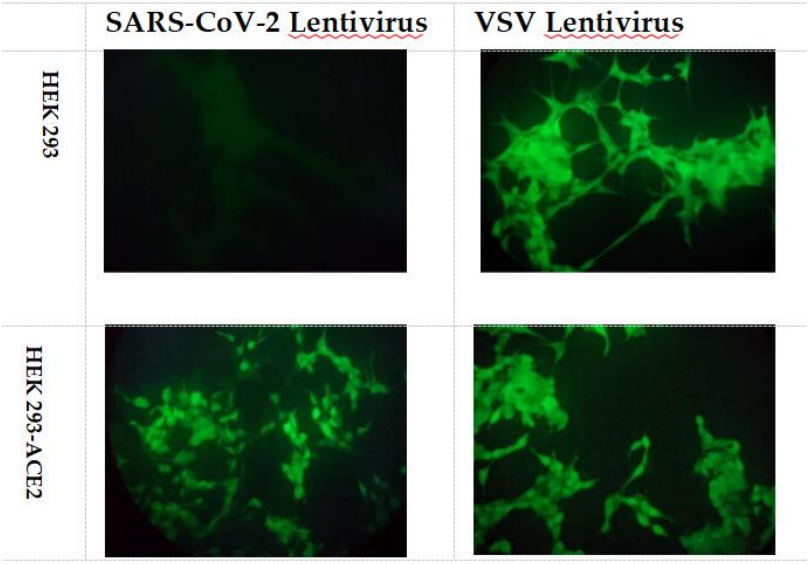


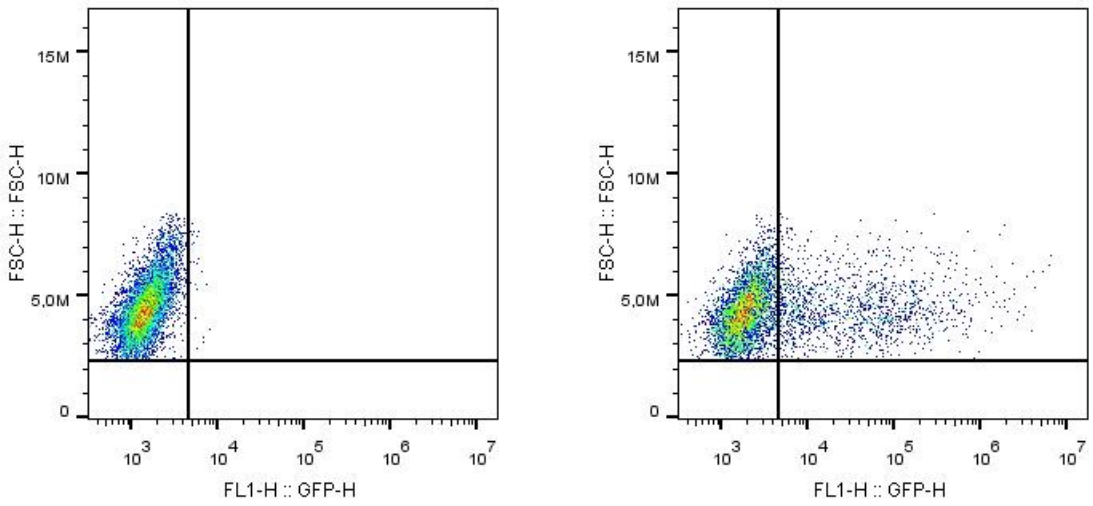
Figure 27. **The Lentivirus transduction resulted in GFP expression, which demonstrated that the SARS-CoV-2 Spike-expressing Lentivirus had a specific binding to HEK 293-ACE2 cells**

4.2.3. Titration of SARS-CoV-2 and VSV lentiviruses

The titration process involved using a lentiviral pseudotype, which expresses the spike protein of SARS-CoV-2, to transfer a reporter molecule (in this case, GFP) into target cells.

In order to find out the titre of lentiviruses, both VSV and SARS-CoV-2 lentiviral preparations were 2-fold serially diluted and 200 µL of each was mixed with $3x10^5$ HEK293-ACE2 cells, and the transduction efficiency was evaluated by flow cytometry 72 hours later. The infection ratios were calculated by observing the presence of GFP protein in the infected cells. This allowed us to distinguish between transduced and non-transduced cells using a green fluorescence signal, as shown in figure 28. The percentage of GFP-positive infected cells (highlighted in table 4) was used to estimate the quantity of virus that was produced, while the values that showed the most linearity along the 2-fold dilution curve were chosen to calculate the titre (figure 29).

Based on this analysis, the SARS-CoV-2 lentivirus preparation had a titre of approximately $3x10^5$ TU/mL, as measured in five independent experiments. This titre was found to be one order of magnitude lower than the titre achieved with VSV lentivirus, which had a titre of approximately $6.69x10^6$ TU/mL. These findings are consistent with previous research reported in the literature, suggesting that the VSV lentivirus preparation was more efficient at transducing cells compared to the SARS-CoV-2 lentivirus preparation (figure 29) [112].



(**a**) (**b**)

Figure 28. **Analysis of green fluorescence by flow cytometry in HEK 293-ACE2 cells under two conditions**

(a) not transduced and (b) transduced with SARS-CoV-2 Lentivirus

Table 4. **Transduction efficiency and titre of lentiviral preparations at various volumes**

Lenti SARS-CoV-2		**Lenti VSV**	
Virus volume	% of transduction	Virus volume	% of transduction
200 µL	**54%**	200 µL	52,46%
100 µL	**32%**	100 µL	63,93%
50 µL	**16%**	50 µL	55,1%
25 µL	13,5%	25 µL	35,11%
12.5 µL	12%	**12.5 µL**	**30,2%**
6 µL	10%	**6 µL**	**18,35%**
3 µL	9%	**3 µL**	**11,58%**
NT	0,2%	NT	0,5%
Titre: 3 x 10^5 TU/ml		**Titre**: 6,69 x 10^6 TU/ml	

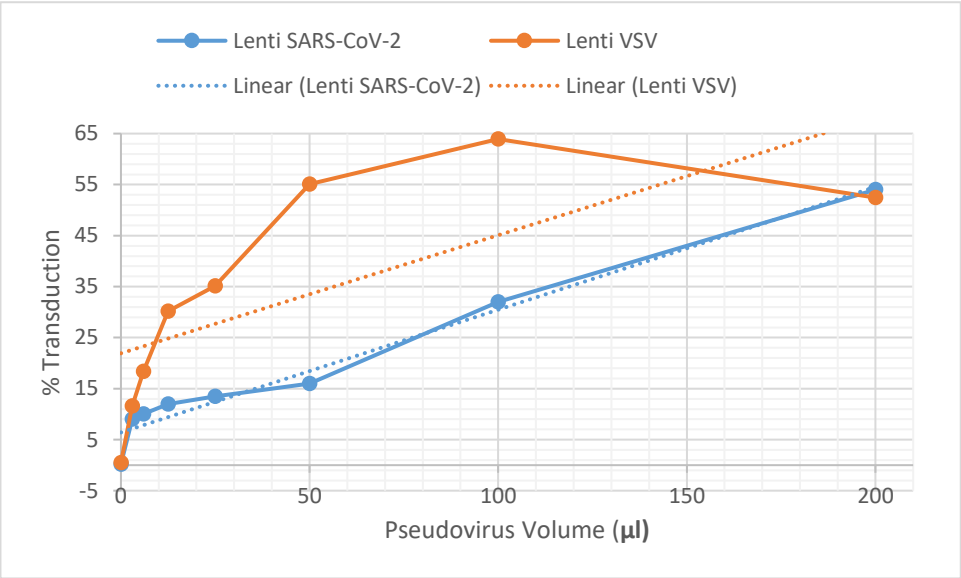


Figure 29. **Titration of LV Spike SARS-CoV-2 and LV VSV on HEK293-ACE2 cells** [17]

Accurate determination of viral titre is essential for ensuring that the appropriate dose of the virus is administered to achieve therapeutic or protective effects. Therefore, this study provides valuable insights into the titration of SARS-CoV-2 and VSV lentiviruses, which could have important implications for developing viral vector-based therapies and vaccines.

In conclusion, this research on the titration of SARS-CoV-2 and VSV lentiviruses using HEK 293-ACE2 cells has provided important insights into the transduction efficiency of these viruses and their potential use in viral vector-based therapies and vaccines. This research findings suggest that VSV lentivirus has a higher transduction efficiency than SARS-CoV-2, which could be important for optimizing the delivery of viral vectors in future therapeutic and vaccine applications.

4.2.4. Analysis of immune responses to SARS-CoV-2 in recovered COVID-19 patients and Sinopharm vaccinated individuals

4.2.4.1. SARS-CoV-2 neutralising antibodies after natural infection or following vaccination

Our next objective was to examine the ability of antibodies produced by either the Sinopharm vaccine or natural infection to neutralise the virus. In order to achieve this goal, we established two separate assays that utilize SARS-CoV-2 pseudotyped lentiviruses. One of these assays involved the use of HCI microscopy, while the other employed FC.

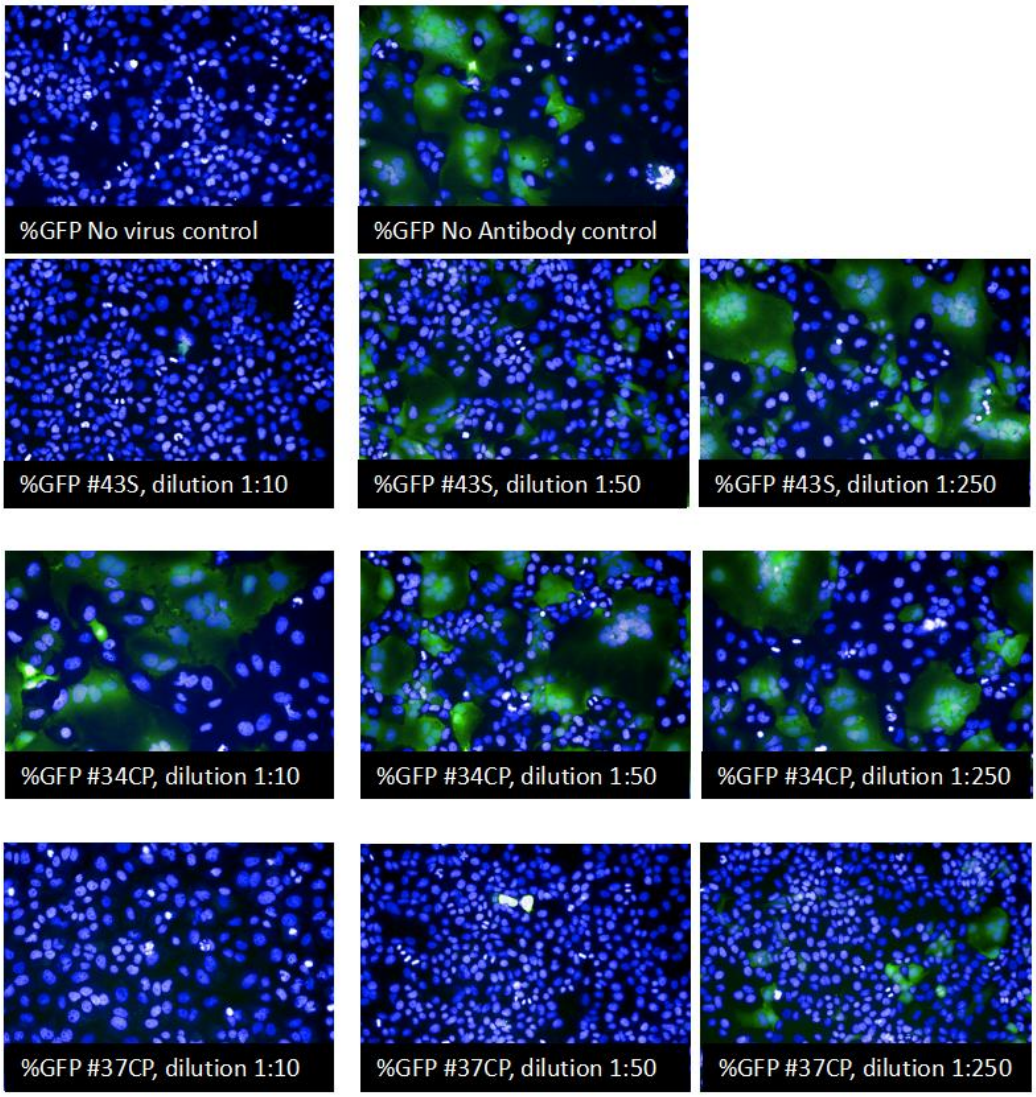


Figure 30. **Examples of images obtained through HCI illustrating the results of the neutralisation assay**

Figure 30 and figure 31 display representative images from two different tests: HCI (figure 30) and FC (figure 31). In the upper two quadrants of the figures, control plots are shown for non-transduced cells (NT) and transduced cells in the presence of pre-Covid sera at the dilution of 1:10. The neutralisation results were analysed using both techniques.

In figure 30, cells that were successfully transduced with the virus were identified by the green fluorescence signal, while the nuclei of all cells were labelled with DAPI, appearing blue. The image is composed of spots, each representing a single cell, with the green spots indicating cells that were infected with the virus.

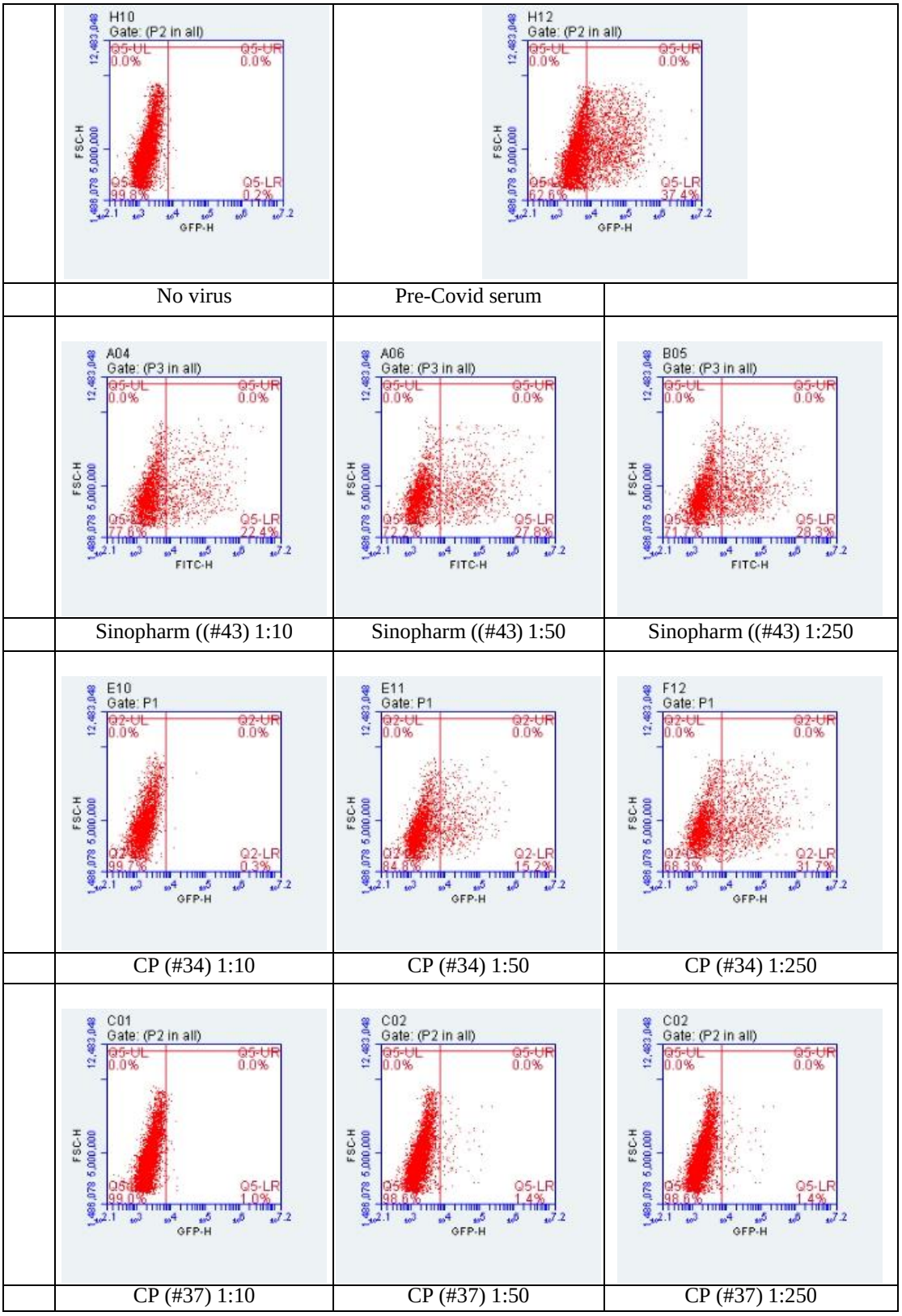


Figure 31. **Detection of SARS-CoV-2 neutralising antibodies by FC: representative assay images**

Figure 31 shows a flow cytometry plot where the numbers indicate the percentage of GFP-positive cells in the different quadrants.

The data presented in both figures are from a single experiment, representing three independent experiments.

The green fluorescence background in the FC analysis for NT cells was determined to be around 0.2% - 0.5%, and SARS-CoV-2 lentivirus with or without pre-Covid sera transduced around 28% - 40% of cells. The HCI tests produced similar results to FC. The negative control displayed 0.04% to 0.2% of positive cells, while the positive control demonstrated a range of GFP-positive cells between 19.80% and 42.01% [17].

By employing two different techniques, it was feasible to categorize all the examined samples into three distinct groups, as illustrated in figures 30 and 31. The first group consisted of non-neutralising or weakly neutralising serum, represented by sample #34CP. The second group was characterized as moderately neutralising serum, represented by sample #43S. Finally, the third group was defined as strongly neutralising serum, represented by sample #37CP. This classification was achieved by applying both methodologies, which provided a comprehensive assessment of the neutralising capacity of the samples.

In the case of convalescent patients, the neutralisation assays showed that only 20% of the samples efficiently neutralised the pseudotyped SARS-CoV-2 at titres above 1:250. This means that a large proportion of convalescent patients had low levels of neutralising antibodies. In fact, for 50% of sera, the response was weak or absent when determined either by flow cytometry or high content imaging, with titres ranging between 1:10-1:50. This suggests that a significant portion of convalescent patients may not have developed a strong humoral immune response to the virus even after two weeks of recovery from the infection. It is possible that these patients may still be at risk of re-infection, which highlights the importance of continued monitoring and public health measures to prevent the spread of the virus.

When comparing the two methods used for neutralisation assays (table 5), flow cytometry showed a median NT_{50} of 27.6 (95% CI: 13.6 - 31.8) (range: 1-1819) in convalescent patients, while high content imaging showed a median titre of 24.6 (95% CI: 11.4 - 29.4) (range: 1-11051). The mean convalescent titre was 357 (95% CI: 0 - 623) when tested by HCI and 123 (95% CI: 61.5 - 175) when determined by FC. This indicates that both methods are comparable in terms of detecting neutralising antibodies in convalescent patients.

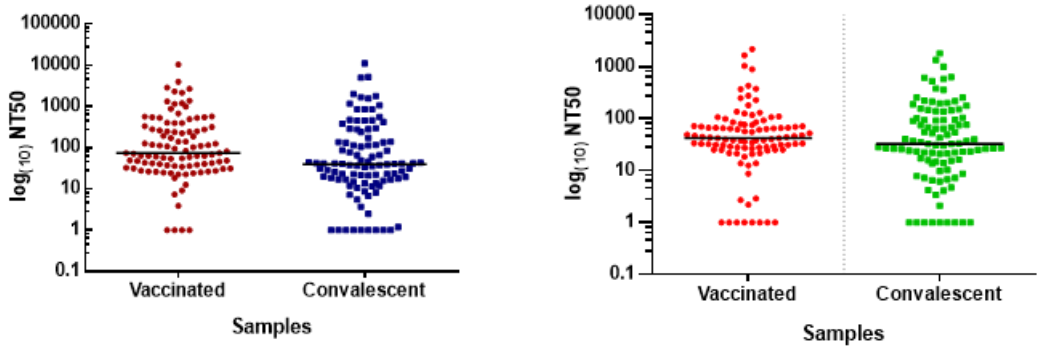
On the other hand, similar to convalescent participants, the neutralisation assays performed on vaccinated individuals using both FC and HCI methods showed that only 20% of the samples efficiently neutralized the pseudotyped SARS-CoV-2 at titres above 1:250. For 50% of the samples, the response was weak or absent when determined either by FC or HCI (titres ranging between 1:10-1:50).

Table 5. **Comparison of quantitative antibody titres between studied groups and subgroups using median and range across methods** [17]

	Overall titre	Titre in convalescent	Titre in vaccinated	Titre in vaccinated (+prior infection)	Titre in vaccinated (naïve)
ELISA IgG RBD; median (range)	1678 (1-13565)	1239 (1-13565)	1742 (152-7184)	1936 (524-6978)	1731 (152-7184)
FC; median (range)	35.8 (1-2182)	27.6 (1-1819)	40.1 (1-2182)	43.9 (1-896)	39.6 (1-2182)
HCI; median (range)	41.3 (1-11051)	24.6 (1-11051)	60.9 (1-10451)	67 (1-3940)	57 (1-10451)

Nonlinear regression analysis was used to determine half-maximal neutralisation titres (NT_{50}), which were found to be 40.1 (95% CI: 27.8 - 47.4) with range values of 1 to 2182 for vaccinated individuals using the FC method. Similarly, for the HCI method group, the study found that the NT50 was 60.9 (95% CI: 40.2 - 78.6) and the range value of 1 to 10451 (range: 1-10451) (table 5). This indicates that high-content imaging may be slightly more sensitive than flow cytometry in detecting neutralising antibodies in vaccinated individuals.

Overall, these results suggest that both convalescent patients and vaccinated individuals may have a suboptimal humoral immune response to SARS-CoV-2. This may have implications for the durability of protection against the virus and the potential need for booster vaccinations in the future.



(**a**) (**b**)

Figure 32. **Neutralising antibody titres in Sinopharm vaccinated cohort, and COVID-19 recovered patients measured by (a) HCI and (b) FC** [17]

Figure 30 shows the neutralising antibody titres in the Sinopharm vaccinated cohort and COVID-19-recovered patients, as measured by high-content screening microscopy (HCI) and flow

cytometry (FC). The black line indicates the median titres. The scatter plot displays the results obtained by both methods. For visual clarity, the 25 extreme values are not shown in the plot but were included in the calculations. The sera from healthy donors were tested at a single 1:12.5 dilution, and all showed an NT50 < 0.94.

Interestingly, there was no significant difference in the level of neutralising antibodies between vaccinated and convalescent patients, suggesting that vaccination may not necessarily provide a superior immune response to natural infection. However, it is important to note that this study only evaluated the humoral immune response and did not investigate other aspects of the immune system, such as cellular immunity. Therefore, further research is needed to determine the overall effectiveness of vaccination in preventing SARS-CoV-2 infection and its associated disease. It is also worth mentioning that the study only included individuals who received the Sinopharm vaccine, and the results may differ for other vaccine types.

4.2.4.2. Comparison of SARS-CoV-2 RBD-specific IgG antibody levels in vaccinated individuals and convalescent patients using ELISA

The RBD-specific ELISA test was conducted to measure the level of IgG anti-Spike RBD antibodies in Sinopharm vaccinated individuals and convalescent patients. The OD threshold for the test was 0.0819 (figure 33), and the IgG titres were defined as the reciprocal of the last dilution at which the OD450 was above the threshold (figure 34).

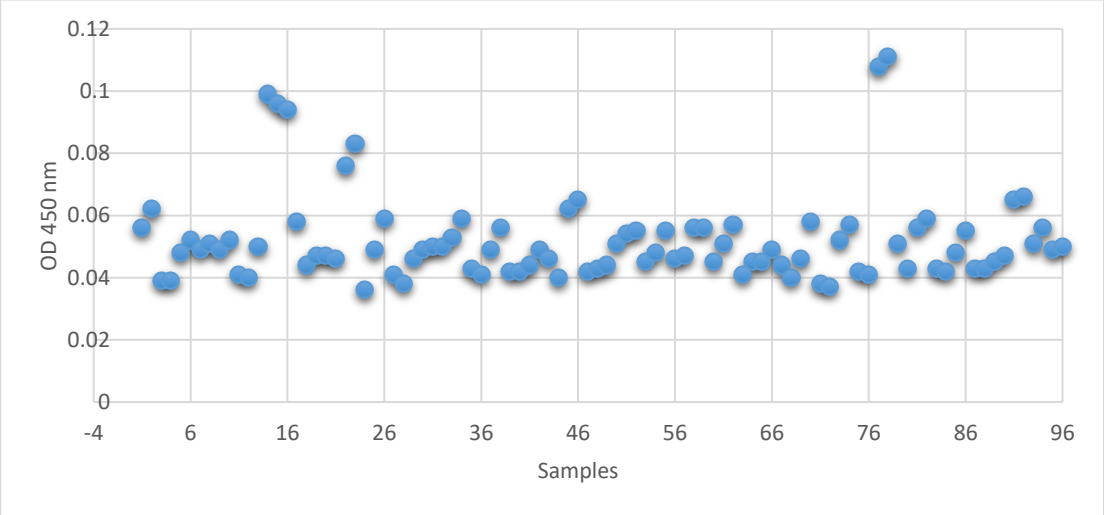


Figure 33. **Cut-off for anti-SARS-CoV-2 Spike RBD IgG in 96 Pre-COVID sera**

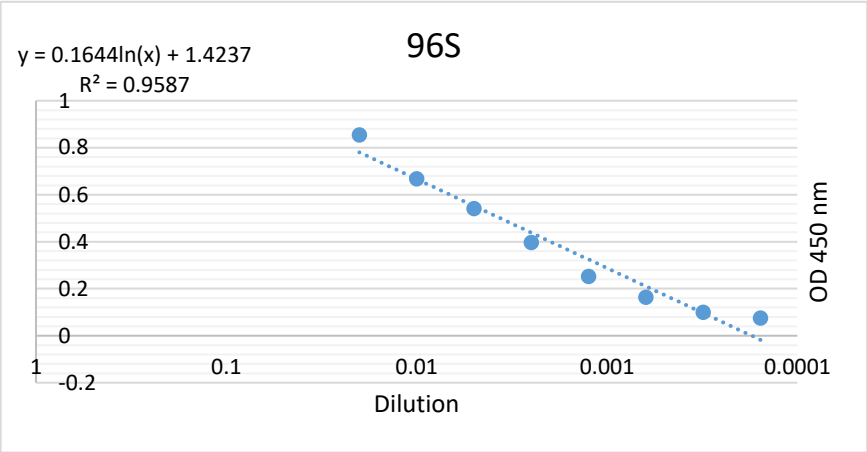


Figure 34. **Determination of IgG titre by threshold value-based method: example of sample 96 from Sinopharm vaccinated recipient**

The results showed that both groups had potent and specific serological activity towards RBD binding compared to pre-pandemic healthy controls (figure 35). However, the Sinopharm vaccination induced a stronger humoral immune response than natural infection, as the vaccinated individuals had significantly higher anti-RBD IgG antibody levels compared to convalescent patients, with median titres of 1742 versus 1239 (table 5). These findings are consistent with previous studies that have reported higher antibody titres in vaccinated individuals compared to those who have recovered from COVID-19 [17].

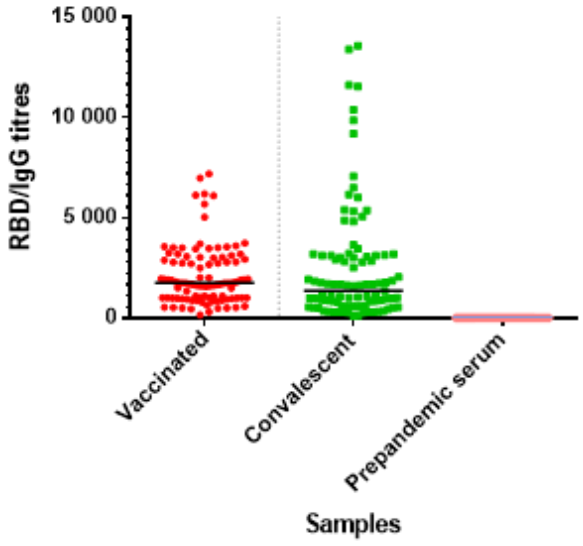


Figure 35. **Comparison of Anti-SARS-CoV-2 RBD IgG antibody levels in Sinopharm-vaccinated, COVID-19 recovered and seronegative individuals**

Figure 35 shows a comparison of anti-SARS-CoV-2 RBD IgG antibody titres in three groups: 100 Sinopharm-vaccinated recipients (red), 100 COVID-19 recovered individuals (green), and 96 seronegative subjects (pink). A horizontal black line represents the median antibody level.

The overall titre (measured in 200 samples) had a mean titre of 2322 (CI: 1991-2653) and a median titre of 1678 (CI: 1598-1863). It exhibited a higher standard deviation (2342) and a wider interquartile range (2151) compared to the convalescent and vaccinated titres. The range of values observed for the overall titre was 1 to 13565.

In the convalescent group (measured in 100 samples), the mean titre was 2519 (CI: 1906-3069) and the median titre was 1239 (CI: 745-1486). It had the highest standard deviation (2959) and interquartile range (2521), with a range of values from 1 to 13565.

For the vaccinated group (measured in 100 samples), the mean titre was 2126 (CI: 1830-2409) and the median titre was 1742 (CI: 1565-1882). It had the lowest standard deviation (1481) and interquartile range (1930), with a range of values from 152 to 7184.

Additionally, SARS-CoV-2 RBD-specific IgG antibodies were present in the sera of all vaccinated subjects, while one convalescent individual (60CP) had undetectable titre values. This individual appears to be a healthy non-responder who did not produce antibodies after recovering from COVID-19 [17].

The results demonstrated that Sinopharm vaccination induced a more robust humoral immune response than natural infection, as the vaccinated individuals had significantly higher anti-RBD IgG antibody levels than convalescent patients. These findings are consistent with previous studies that have reported higher antibody titres in vaccinated individuals compared to those who have recovered from COVID-19. It is worth noting that the neutralising antibody response is also higher in vaccinated individuals than in convalescent patients.

The differences in antibody levels between vaccinated and convalescent individuals could potentially be due to several factors, including differences in the quality and quantity of the immune response generated by the vaccine versus natural infection. Other studies have also reported higher antibody levels in vaccinated individuals compared to convalescent individuals, which is consistent with our findings. However, further research is needed to understand these differences' underlying mechanisms fully.

Our findings have important implications for developing effective treatments and vaccines for COVID-19. Specifically, they suggest that vaccination with the Sinopharm vaccine can result in higher levels of anti-RBD SARS-CoV-2 IgG antibodies compared to natural infection, which may confer greater protection against the virus. However, it is important to note that antibody levels alone do not necessarily predict protection against COVID-19, and other aspects of the

immune response, such as T-cell immunity, should also be considered in developing effective treatments and vaccines.

4.2.4.3. Correlation between neutralising and anti-RBD SARS-CoV-2 IgG antibodies

The spike receptor-binding domain (RBD) of the SARS-CoV-2 virus is a critical component in triggering the production of neutralising antibodies (nAbs) that can help protect against the virus. The RBD also significantly impacts the activation of T-cell immune responses, further emphasizing its importance in the body's defence against COVID-19 [174].

Table 6. **Correlation between SARS-CoV-2-specific antibody responses and neutralisation titres using different methods**

Group	method_1	method_2	correlation_coefficient (spearman)	p_val
All	ELISA_RBD	FC	0,64	< 0.001
Convalescent	ELISA_RBD	FC	0,68	< 0.001
Vaccinated	ELISA_RBD	FC	0,58	< 0.001
All	HCI	ELISA_RBD	0,52	< 0.001
Convalescent	HCI	ELISA_RBD	0,45	< 0.001
Vaccinated	HCI	ELISA_RBD	0,53	< 0.001
All	FC	HCI	0,55	< 0.001
Convalescent	FC	HCI	0,51	< 0.001
Vaccinated	FC	HCI	0,58	< 0.001

The results of the correlation analysis between the neutralising antibodies obtained by FC or HCI assays and anti-RBD SARS-CoV-2 IgG antibodies as measured by ELISA are shown in the table 6. Spearman's rank correlation coefficient was used to evaluate the relationship between the two parameters. There was found moderate to strong positive correlation between the neutralising and anti-RBD SARS-CoV-2 IgG antibodies, with correlation coefficients ranging from 0.45 to 0.68 and *p*-values less than 0.001 for all comparisons. This suggests that higher levels of anti-RBD SARS-CoV-2 IgG antibodies are associated with a stronger neutralising response to the virus.

Figure 36 compares log10 transformed ELISA RBD and FC titres obtained from convalescent (red) and vaccinated (blue) participants. The figure highlights the best-fit linear regression line and corresponding confidence interval as a black line with a grey band. The different color points indicate the respective participants.

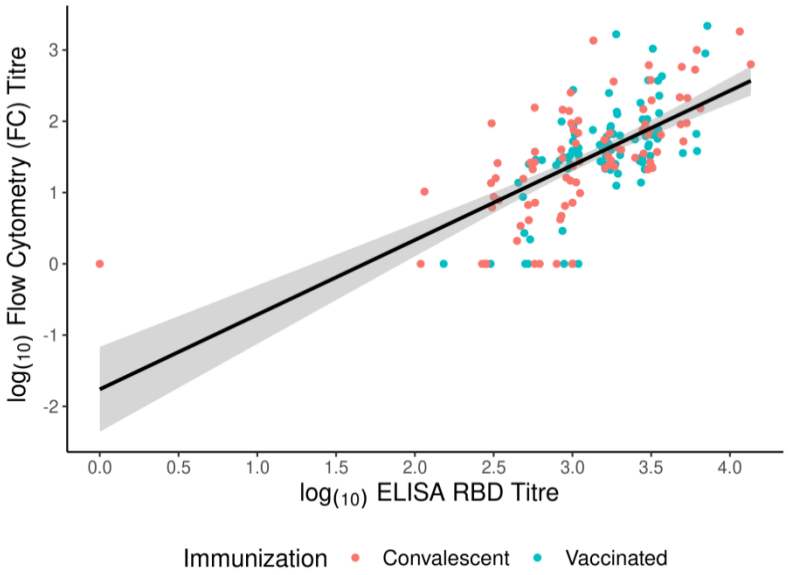


Figure 36. **Comparison of overall log10 transformed ELISA RBD and FC titres** [17]

Specifically, the correlation coefficients between ELISA RBD and FC assays were 0.64 for all participants, as shown in figure 36, while between ELISA RBD and HCI assays, the correlation coefficients were the lowest (ρ=0.52) for all participants, the results being presented in figure 38. These results suggest that while there is a strong relationship between binding and neutralising antibodies, the relationship is not linear and may vary depending on the test used.

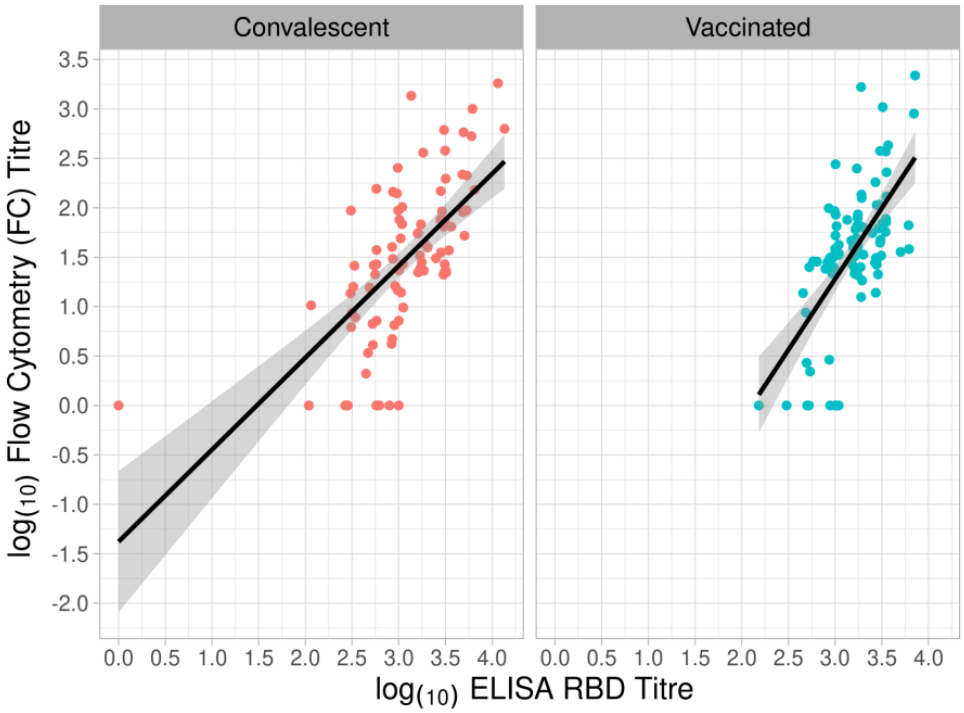


Figure 37. **Correlation between log10 transformed ELISA RBD and FC titres in convalescent versus vaccinated individuals** [17]

Figure 37 illustrates the correlation between log10 transformed ELISA RBD and FC titres in convalescent (red) and vaccinated (blue) individuals. The figure highlights the best-fit linear regression line and corresponding confidence interval as a black line with a grey band. The different color points indicate the respective groups.

Furthermore, when we compared the correlation coefficients between the tests in convalescent and vaccinated groups separately, we found similar results, suggesting that the correlation was not influenced by the type of immune response (i.e., natural infection *vs.* vaccination). The correlation coefficients between ELISA RBD and FC assays were 0.68 for convalescent patients, and 0.58 for vaccinated individuals (figure 37).

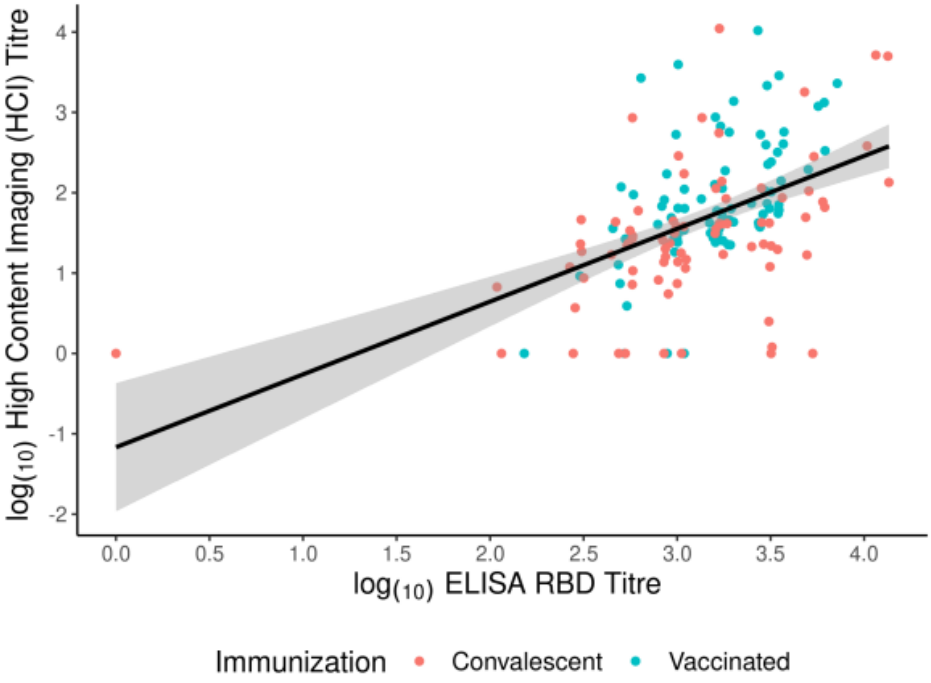


Figure 38. **Correlation between anti-RBD IgG antibodies and HCI neutralising titres in human sera** [17]

Figure 38 displays the correlation between anti-RBD IgG antibodies and neutralising titres in both convalescent (red) and vaccinated (blue) participants. The log10 transformed values of titres obtained from ELISA RBD and NT50 determined by HCI are represented by different colour points. The best-fit linear regression line and corresponding confidence interval are presented as a black line with a grey band.

The correlation coefficients between neutralising antibodies detected by HCI and the anti-SARS-CoV-2 Spike RBD antibodies titres determined by ELISA assay were slightly higher in the vaccinated individuals (ρ=0.53) than in the convalescent patients (ρ=0.45). We found a statistically significant difference between the groups only in the HCI assay ($p < 0.001$), with the median titre

of vaccinated individuals being significantly higher than that of convalescent patients. This result is consistent with previous publications showing that vaccinated individuals have higher antibody titres than convalescent patients [17].

Lastly, the correlation coefficients between FC and HCI assays were 0.55 for all participants, 0.51 for convalescent patients, and 0.58 for vaccinated individuals.

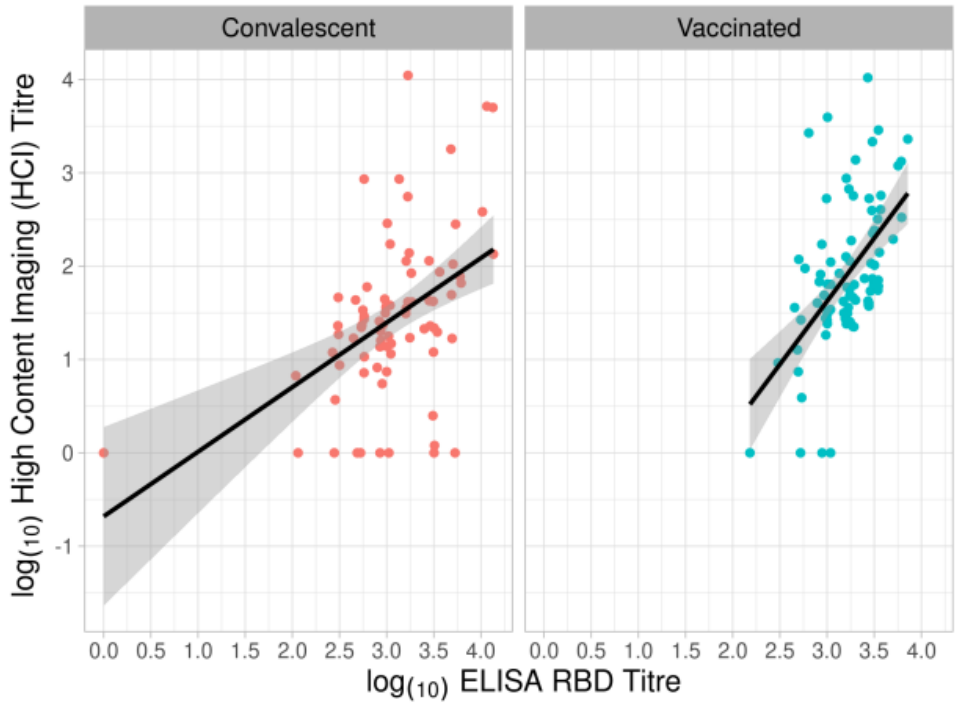
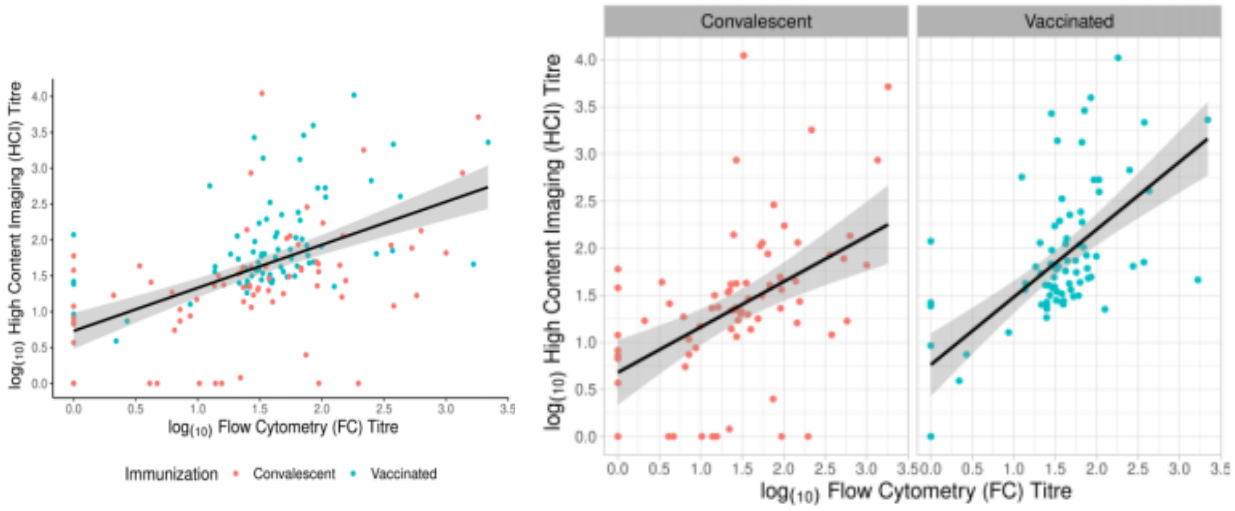


Figure 39. **Anti-RBD IgG antibodies in human sera and its correlation with NT_{50} determined by HCI**

The plot in figure 39 illustrates the relationship between anti-RBD IgG and neutralising antibody levels in convalescent and vaccinated subjects. The graph includes different colour points indicating convalescent (red) and vaccinated (blue) participants. The black line represents the best estimate of the relationship between these two variables, while the grey band represents the range of values the genuine relationship will likely fall within. The fact that there is a best-fit linear regression line with a corresponding confidence interval suggests a correlation between the levels of anti-RBD IgG and neutralising antibodies in these individuals.

Interestingly, we observed that there were some samples with undetectable nAbs that still had binding antibodies, indicating that a large proportion of antibodies do not neutralize the virus. However, the higher the binding titre, the more likely that neutralisation is detected, suggesting that binding and neutralisation do correlate to some extent. Our findings suggest that measuring neutralising and binding antibodies is important for comprehensively evaluating the humoral immune response to SARS-CoV-2.



(**a**) (**b**)

Figure 40. **Correlation of neutralising antibody titres in convalescent and vaccinated groups, determined by FC and HCI**

Figure 40 displays the correlation of each studied group's neutralising antibody titres defined between FC and HCI. The figure is divided into two parts, with the first part showing the log10 transformed values of NT50 determined in both studied groups. The graph likely depicts the relationship between the neutralising antibody titres obtained from the two different assays in each studied group. The second part of the figure shows the correlation of neutralising antibody levels between the two neutralisation assays in convalescent and vaccinated subjects, respectively. Different colour points indicate convalescent (red) and vaccinated (blue) participants, and the best fit linear regression line with the corresponding confidence interval is presented as a black line with a grey band. The steep, upward-sloping line shows a strong positive correlation between the two assays.

Individuals who have had both previous SARS-CoV-2 infection and have received a vaccine showed higher antibody levels compared to those who only had one or the other. The difference was statistically significant only in the HCI titres between convalescent and vaccinated individuals ($p<0.05$). Interestingly, there was no statistical significance in the comparison between those who had recovered from the infection and those who had received the vaccine but never contracted the virus. Furthermore, convalescent individuals and those who were both previously exposed to the virus and vaccinated had significantly higher antibody levels compared to those who had only received the vaccine ($p < 0.001$). This suggests that prior exposure to the virus could lead to a stronger immune response after vaccination, as indicated by the higher titres observed in convalescent individuals and those previously exposed to the virus.

Figure 41 provides a detailed comparison of antibody levels between these different subgroups of participants, and shows the distribution of antibody titres for each group.

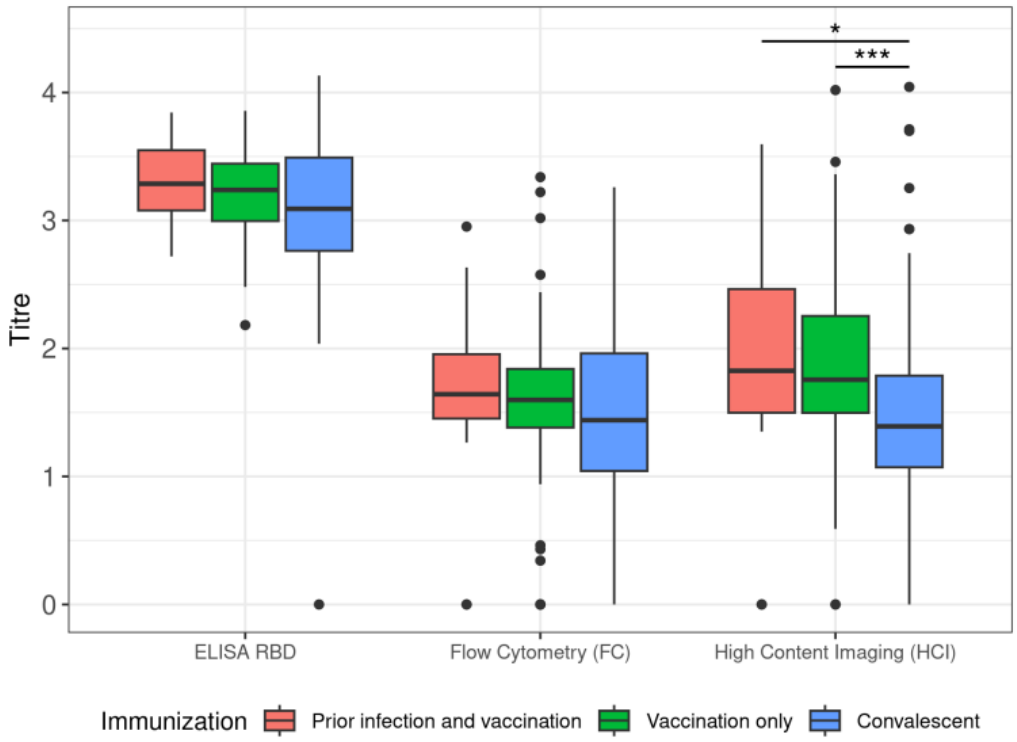


Figure 41. **The relationship between SARS-CoV-2 exposure and vaccine-induced antibody responses**

Our study provides important insights into the correlation between neutralising and anti-RBD SARS-CoV-2 IgG antibodies. These findings have important implications for developing effective treatments and vaccines against COVID-19, as they suggest that a strong humoral immune response, including neutralising and binding antibodies, may be necessary for protection against the virus. However, further research is needed to clarify the exact nature of the relationship between binding and neutralising antibodies, confirm this relationship, evaluate the long-term protective immunity conferred by these antibodies, and determine the most effective methods for measuring the humoral immune response to SARS-CoV-2.

4.3. Discussions

The study discussed above provides valuable insights into the antibody response to the Sinopharm vaccine and natural immunity against SARS-CoV-2. Our study also supports previous reports that donors who completed two doses of vaccines had higher RBD antibody levels than the convalescent group [175]. However, our study focused on the Sinopharm vaccine, which has limited information on its efficacy. The results of this study suggest that the Sinopharm/BBIBP-CorV vaccine may have lower levels of nAbs compared to other more widely used vaccines [176,177]. While 20% of the participants in the study developed high neutralisation titres, most

had moderate to low levels of nAbs. These findings are consistent with previous studies [159,178,179], which also reported lower levels of nAbs in individuals who received the Sinopharm vaccine. However, it is promising to see that all of the immunized human subjects in the study generated IgG RBD antibody responses.

It is also important to note that while most COVID-19 recovered patients and Sinopharm vaccinated subjects developed anti-RBD antibodies, only a small number were able to block pseudotyped SARS-CoV-2 lentivirus from attaching to the hACE2 receptor [17]. These findings suggest that the level of nAbs is an important factor in determining the effectiveness of antibodies in neutralising the virus [177]. The moderate correlation between anti-RBD antibody levels and neutralisation activity observed in this study contrasts with other studies reporting strong correlations ($\rho = 0.875 – 0.819$, $p < 0.001$) between anti-S and neutralising antibody titres [180]. According to another research, there was a significant correlation between the quantity of IgG RBD SARS-CoV-2 antibodies and nAbs [181].These differences in findings may be attributed to variations in study design, sample size, and differences in assay protocols. This suggests that further research is needed to better understand this relationship.

The development of an IgG ELISA with recombinant SARS-CoV-2 Spike RBD and BSL-2 safe assays is a valuable contribution to the field of SARS-CoV-2 research, as high throughput approaches are important for drug discovery and neutralisation assays. The use of pseudoviruses in neutralisation assays has become a common and useful approach for the evaluation of neutralising antibodies in vitro [17,26,173]. The use of high-throughput approaches, such as HCI, can also improve the efficiency of neutralisation assays and reduce costs [183,184]. By modifying a 96-well high content imaging (HCI) technique for SARS-CoV-2, our study provided a more efficient alternative to flow cytometry (FC) for screening neutralising antibodies. The HCI approach allowed for the simultaneous screening of 15 samples, while FC is time-consuming and requires several analysis steps. Therefore, our modified HCI technique provides a more streamlined and practical approach for screening large numbers of samples for the presence of neutralising antibodies against SARS-CoV-2 [17].

This research contributes to the growing body of literature on the immune response to SARS-CoV-2 and the effectiveness of different COVID-19 vaccines and convalescent plasma.

Our findings suggest that the Sinopharm vaccine induces a robust antibody response, which may confer protection against SARS-CoV-2 infection. However, the lower neutralisation titres observed in this study raise questions about the vaccine's effectiveness against emerging variants of the virus. Our study has some limitations, including a relatively small sample size and a cross-sectional design that limits our ability to assess the durability of vaccine-induced immunity and its effectiveness against emerging variants. Additionally, our study focused only on the RBD-ACE2

binding pathway, and other immune functions, such as complement activation and antibody-dependent cellular cytotoxicity, were not assessed but should be studied to gain a more comprehensive understanding of the immune response to SARS-CoV-2.

Overall, this study underscores the importance of continuing research into the development of effective therapies and vaccines against SARS-CoV-2, particularly as new variants of the virus continue to emerge. Furthermore, developing diagnostic tools that are cost-effective and easy to use is critical for countries with limited financial resources. These tools can help these countries to improve their response to outbreaks and epidemics, particularly in remote or rural areas where access to healthcare facilities may be limited. Moreover, developing affordable diagnostic tools that can be used in low-resource settings will also help to ensure equitable access to healthcare for all, regardless of socioeconomic status. Therefore, there is a need for continued investment in the development of diagnostic tools that are both effective and affordable for use in resource-limited settings.

CONCLUSIONS

1. SARS-CoV-2 undergoes a continuous molecular evolution, as evidenced by the presence of several amino acid substitutions in viral proteins in the samples analysed from the Republic of Moldova. Some of these mutations are frequent and may have implications on the transmissibility, virulence and immune escape of the virus, such as Spike D614G, N G204R, N R203K, L452R and P681H mutations.
2. Ongoing monitoring of the molecular evolution of SARS-CoV-2 is necessary to identify emerging mutations and their potential impact on the effectiveness of vaccines and treatments.
3. The SARS-CoV-2 pseudotype lentivirus was successfully produced using a 2nd generation HIV-LV system, which could be used to identify antibodies that can detect the spike protein in its natural form.
4. The neutralisation assays showed that only 20% of the samples in population under study efficiently neutralised the pseudo-type SARS-CoV-2 at titres above 1:250, indicating that a significant portion of convalescent and vaccinated individuals possibly did not have a developed strong humoral immune response to the virus.
5. The Sinopharm COVID-19 vaccine induces a robust and specific immune response in vaccinated individuals, as shown by the high levels of anti-RBD IgG antibodies detected in serum samples.
6. The humoral immune response induced by the Sinopharm vaccine is stronger than the response observed in individuals who have recovered from COVID-19, as indicated by the higher levels of anti-RBD IgG antibodies detected in vaccinated individuals compared to convalescent patients.
7. There is a moderate positive correlation between the levels of anti-RBD SARS-CoV-2 IgG antibodies and the neutralising response to the virus. This suggests that higher levels of anti-RBD SARS-CoV-2 IgG antibodies are associated with a stronger neutralising response to the virus.
8. The study demonstrated the significance of developing diagnostic tools and conducting studies on the humoral immune response to fight against the transmission of SARS-CoV-2 and mitigate its impact on public health effectively. This is especially important for the Republic of Moldova, as such efforts can provide invaluable insights into the molecular epidemiology of the virus and contribute to the global fight against the pandemic.

RECOMENDATIONS

1. It is crucial to strengthen and enhance the effectiveness of surveillance systems to monitor the molecular evolution of SARS-CoV-2. Regular monitoring should be conducted to track the prevalence of mutations, especially those with implications for transmissibility, virulence, and immune escape.

2. Scientific research should continue to develop diagnostic tools that can accurately measure the humoral immune response to SARS-CoV-2. By measuring both neutralising and binding antibodies, a comprehensive evaluation of the immune response can be achieved. This will provide valuable insights into the effectiveness of vaccines, identify individuals with weak immune responses, and assist in the development of targeted therapies.

3. Given the global impact of the pandemic, regional and international collaboration and sharing of scientific data and findings are crucial. The Republic of Moldova should actively participate in global initiatives to combat the transmission of SARS-CoV-2. Sharing insights from studies conducted in the country can contribute to the global fight against the pandemic and assist in the development of effective containment and treatment strategies.

4. It is recommended to implement the developed protocols and tools from this research project into the teaching and practical curriculum of the microbiology department. This will provide students with hands-on experience and exposure to cutting-edge research methodologies, preparing them for future scientific endeavours and equipping them with knowledge and skills to be prepared and response to potential future pandemic.

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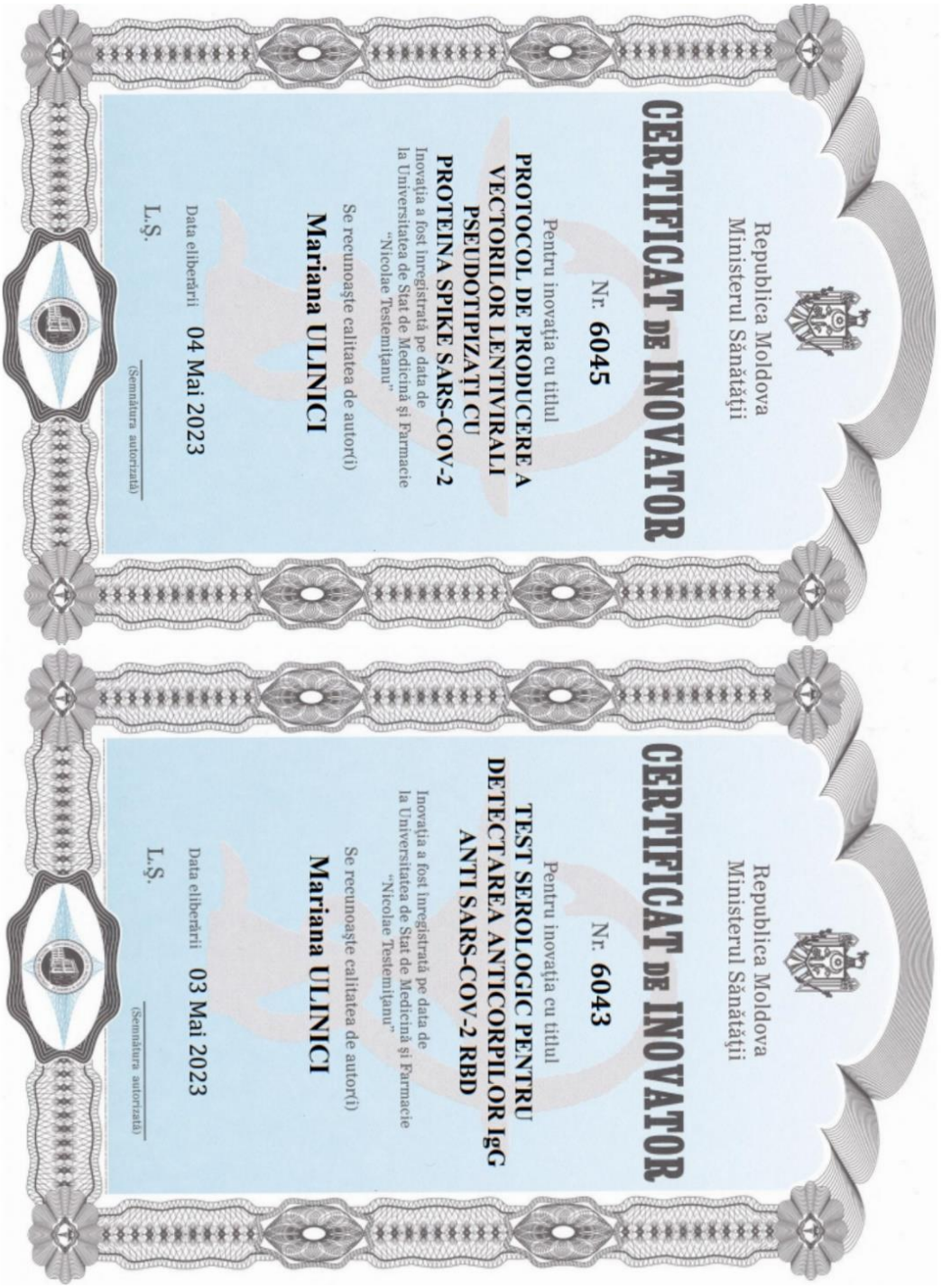
ANEXES

Anexa 1.

Certificat de Inovator Nr. 6043 din 03.05.2023
„Test serologic pentru detectarea anticorpilor IgG anti SARS-COV-2 RBD”
Certificat de Inovator Nr. 6045 din 04.05.2023
„Protocol de producere a vectorilor lentivirali pseudotipizați cu proteina Spike SARS-CoV2”



Anexa 2.

Act de implementare nr. 01-4/78 din 05 mai 2023
Protocol de producere a vectorilor lentivirali pseudotipizați cu proteina Spike SARS-CoV-2.

MINISTERUL SĂNĂTĂȚII
INSTITUȚIA MEDICO-SANITARĂ PUBLICĂ
SPITALUL CLINIC DE BOLI INFECȚIOASE "TOMA CIORBĂ"
Republica Moldova, Chișinău, Ștefan cel Mare și Sfânt, 163, MD-2004
www.tomaciorba.md, tomaciorba@ms.md
+373 (22) 24-22-47, +373 (22) 24-21-77

Nr. 01-4/78 din "05" 05 2023

APROB
Director IMSP
SCBI „Toma Ciorbă"
S. VASILIȚA

ACT DE IMPLEMENTARE

1. **Denumirea ofertei pentru implementare: „PROTOCOL DE PRODUCERE A VECTORILOR LENTIVIRALI PSEUDOTIPIZAȚI CU PROTEINA SPIKE SARS-COV-2".**
2. **Autori:** Mariana ULINICI, asistent universitar,
3. **Numărul inovației:** ***Nr.6045 din 04 mai 2023.***
4. **Unde și când a fost implementată:** Laboratorul bacteriologic IMSP SCBI „Toma Ciorbă".
5. **Eficacitatea implementării:** Inovația propusă este importantă deoarece permite producerea de vectori lentivirali pseudotipizați cu proteina spike a virusului SARS-CoV-2, ceea ce facilitează studierea răspunsului imun la această proteină și dezvoltarea de vaccinuri și terapii împotriva COVID-19. În plus, utilizarea acestui protocol poate fi mai sigură și mai ușor de gestionat decât lucrul direct cu virusul SARS-CoV-2, ceea ce poate reduce riscurile și costurile asociate. Această inovație poate rezolva o serie de probleme asociate cu studierea și dezvoltarea de vaccinuri și terapii împotriva COVID-19, oferind o metodă mai eficientă și mai sigură de a studia comportamentul virusului, fără a fi nevoie să se lucreze direct cu virusul SARS-CoV-2.
6. **Rezultatele:** Implementarea acestui protocol ar putea duce la îmbunătățirea cunoștințelor și înțelegerii despre modul în care proteina spike a virusului SARS-CoV-2 interacționează cu celulele umane și despre modul în care sistemul imun răspunde la această proteină. Acest lucru poate facilita dezvoltarea de vaccinuri și terapii împotriva COVID-19 și îmbunătăți capacitatea de a preveni și trata această boală ceea ce ar putea avea un impact semnificativ asupra sănătății publice la nivel global. Implementarea acestui protocol ar putea, de asemenea, accelera procesul de cercetare prin simplificarea studiilor și reducerea riscurilor și costurilor implicate în manipularea virusului SARS-CoV-2. Propunerea este adresată medicilor de profil microbiologic și este utilizat în practica de laborator în IMSP SCBI „Toma Ciorbă".

Prezenta inovație este implementată conform descrierii în cerere.

Medic bacteriolog,
IMSP SCBI „Toma Ciorbă" COTOS Viorica

Anexa 3.

Act de implementare nr. 01-4/79 din 05 mai 2023.
Test serologic pentru detectarea anticorpilor IgG anti SARS-COV-2 RBD.

MINISTERUL SĂNĂTĂȚII
INSTITUȚIA MEDICO-SANITARĂ PUBLICĂ
SPITALUL CLINIC DE BOLI INFECȚIOASE "TOMA CIORBĂ"

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www.tomaciorba.md, tomaciorba@ms.md
+373 (22) 24-22-47, +373 (22) 24-21-77

Nr. 01-4/79 din "05" 05 2023

APROB
Director IMSP
SCBI „Toma Ciorbă"
S. VASILIȚA

ACT DE IMPLEMENTARE

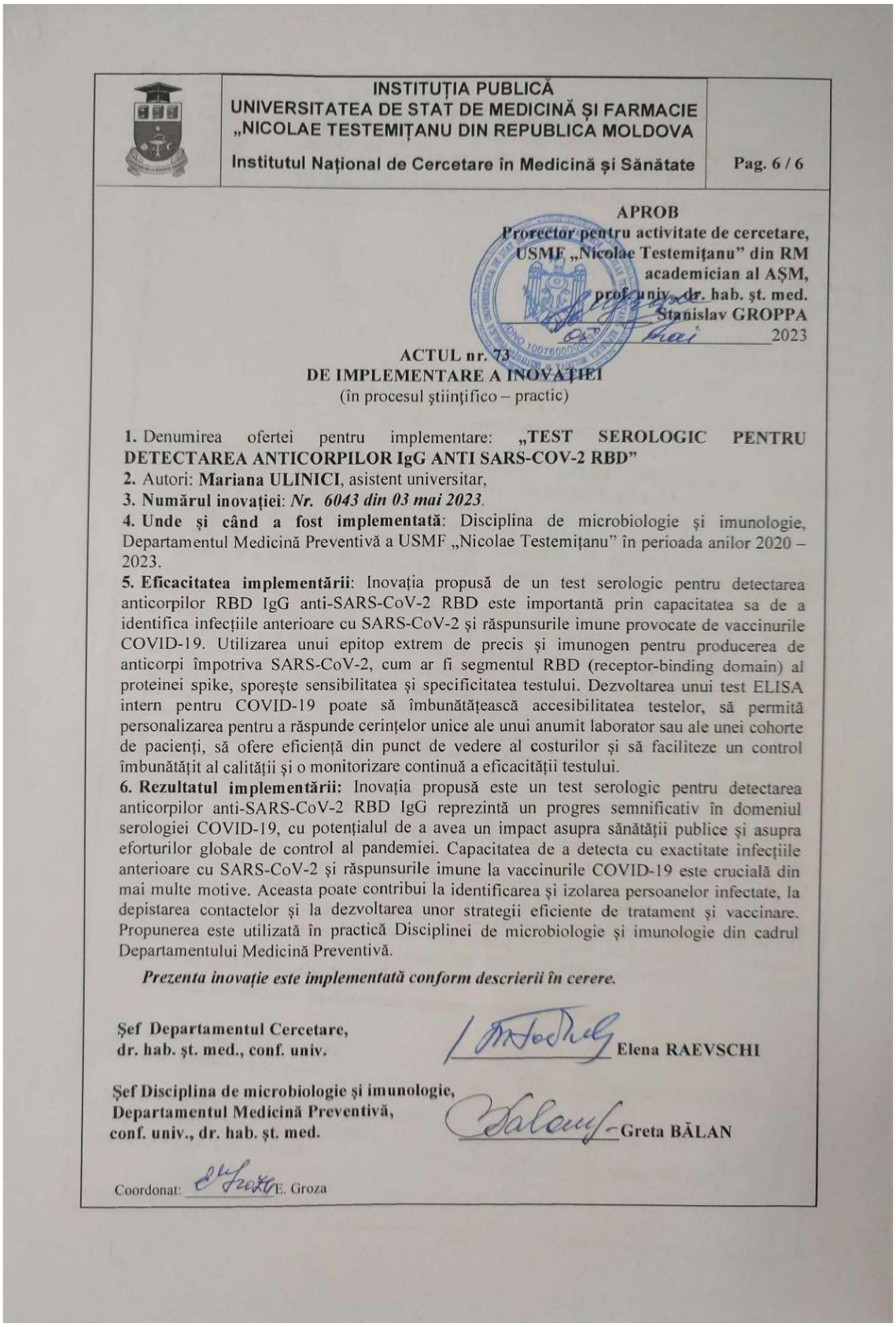
1. **Denumirea ofertei pentru implementare: „TEST SEROLOGIC PENTRU DETECTAREA ANTICORPILOR IgG ANTI SARS-COV-2 RBD".**
2. **Autori:** Mariana ULINICI, asistent universitar,
3. **Numărul inovației:** ***Nr. 6043 din 03 mai 2023.***
4. **Unde și când a fost implementată:** Laboratorul bacteriologic IMSP SCBI „Toma Ciorbă".
5. **Eficacitatea implementării:** Inovația propusă de un test serologic pentru detectarea anticorpilor RBD IgG anti-SARS-CoV-2 RBD este importantă prin capacitatea sa de a identifica infecțiile anterioare cu SARS-CoV-2 și răspunsurile imune provocate de vaccinurile COVID-19. Utilizarea unui epitop extrem de precis și imunogen pentru producerea de anticorpi împotriva SARS-CoV-2, cum ar fi segmentul RBD (receptor-binding domain) al proteinei spike, sporește sensibilitatea și specificitatea testului. Dezvoltarea unui test ELISA intern pentru COVID-19 poate să îmbunătățească accesibilitatea testelor, să permită personalizarea pentru a răspunde cerințelor unice ale unui anumit laborator sau ale unei cohorte de pacienți, să ofere eficiență din punct de vedere al costurilor și să faciliteze un control îmbunătățit al calității și o monitorizare continuă a eficacității testului.
6. **Rezultatul implementării:** Inovația propusă este un test serologic pentru detectarea anticorpilor anti-SARS-CoV-2 RBD IgG reprezintă un progres semnificativ în domeniul serologiei COVID-19, cu potențialul de a avea un impact asupra sănătății publice și asupra eforturilor globale de control al pandemiei. Capacitatea de a detecta cu exactitate infecțiile anterioare cu SARS-CoV-2 și răspunsurile imune la vaccinurile COVID-19 este crucială din mai multe motive. Aceasta poate contribui la identificarea și izolarea persoanelor infectate, la depistarea contactelor și la dezvoltarea unor strategii eficiente de tratament și vaccinare. Propunerea este adresatè medicilor de profil microbiologic și este utilizat în practica de laborator în IMSP SCBI „Toma Ciorbă".

Prezenta inovație este implementată conform descrierii în cerere.

Medic bacteriolog,
IMSP SCBI „Toma Ciorbă" COTOS Viorica

Anexa 4.

Act de implementare nr. 73 din 05.05.2023..
Test serologic pentru detectarea anticorpilor IgG anti SARS-COV-2 RBD.



INSTITUȚIA PUBLICĂ
UNIVERSITATEA DE STAT DE MEDICINĂ ȘI FARMACIE
„NICOLAE TESTEMIȚANU DIN REPUBLICA MOLDOVA
Institutul Național de Cercetare în Medicină și Sănătate

APROB
Prorector pentru activitate de cercetare,
USMF „Nicolae Testemițanu" din RM
academician al AȘM,
prof. univ., dr. hab. șt. med.
Stanislav GROPPA
05 Mai 2023

ACTUL nr. 73
DE IMPLEMENTARE A INOVAȚIEI
(în procesul științifico – practic)

1. Denumirea ofertei pentru implementare: **„TEST SEROLOGIC PENTRU DETECTAREA ANTICORPILOR IgG ANTI SARS-COV-2 RBD"**
2. Autori: **Mariana ULINICI**, asistent universitar,
3. Numărul inovației: ***Nr. 6043 din 03 mai 2023.***
4. Unde și când a fost implementată: Disciplina de microbiologie și imunologie, Departamentul Medicină Preventivă a USMF „Nicolae Testemițanu" în perioada anilor 2020 – 2023.
5. Eficacitatea implementării: Inovația propusă de un test serologic pentru detectarea anticorpilor RBD IgG anti-SARS-CoV-2 RBD este importantă prin capacitatea sa de a identifica infecțiile anterioare cu SARS-CoV-2 și răspunsurile imune provocate de vaccinurile COVID-19. Utilizarea unui epitop extrem de precis și imunogen pentru producerea de anticorpi împotriva SARS-CoV-2, cum ar fi segmentul RBD (receptor-binding domain) al proteinei spike, sporește sensibilitatea și specificitatea testului. Dezvoltarea unui test ELISA intern pentru COVID-19 poate să îmbunătățească accesibilitatea testelor, să permită personalizarea pentru a răspunde cerințelor unice ale unui anumit laborator sau ale unei cohorte de pacienți, să ofere eficiență din punct de vedere al costurilor și să faciliteze un control îmbunătățit al calității și o monitorizare continuă a eficacității testului.
6. Rezultatul implementării: Inovația propusă este un test serologic pentru detectarea anticorpilor anti-SARS-CoV-2 RBD IgG reprezintă un progres semnificativ în domeniul serologiei COVID-19, cu potențialul de a avea un impact asupra sănătății publice și asupra eforturilor globale de control al pandemiei. Capacitatea de a detecta cu exactitate infecțiile anterioare cu SARS-CoV-2 și răspunsurile imune la vaccinurile COVID-19 este crucială din mai multe motive. Aceasta poate contribui la identificarea și izolarea persoanelor infectate, la depistarea contactelor și la dezvoltarea unor strategii eficiente de tratament și vaccinare. Propunerea este utilizată în practică Disciplinei de microbiologie și imunologie din cadrul Departamentului Medicină Preventivă.

Prezenta inovație este implementată conform descrierii în cerere.

Șef Departamentul Cercetare,
dr. hab. șt. med., conf. univ. **Elena RAEVSCHI**

Șef Disciplina de microbiologie și imunologie,
Departamentul Medicină Preventivă,
conf. univ., dr. hab. șt. med. **Greta BĂLAN**

Coordonat: E. Groza

Anexa 5.

Act de implementare nr. 75 din 05.05.2023..

Protocol de producere a vectorilor lentivirali pseudotipizați cu proteina Spike SARS-CoV-2.

INSTITUȚIA PUBLICĂ
UNIVERSITATEA DE STAT DE MEDICINĂ ȘI FARMACIE
„NICOLAE TESTEMIȚANU DIN REPUBLICA MOLDOVA
Institutul Național de Cercetare în Medicină și Sănătate

Pag. 9 / 9

APROB
Prorector pentru activitate de cercetare,
USMF „Nicolae Testemițanu" din RM
academician al AȘM,
prof. univ., dr. hab. șt. med.
Stanislav GROPPA
"___" _____ 2023

ACTUL nr. 75
DE IMPLEMENTARE A INOVAȚIEI
(în procesul ştiinţifico – practic)

7. Denumirea ofertei pentru implementare: **„PROTOCOL DE PRODUCERE A VECTORILOR LENTIVIRALI PSEUDOTIPIZAȚI CU PROTEINA SPIKE SARS-COV-2"**
8. Autori: **Mariana ULINICI**, asistent universitar,
9. **Numărul inovației**: ***Nr.6045 din 04 mai 2023***..
10. **Unde și când a fost implementată**: Disciplina de epidemiologie, Departamentul Medicină Preventivă a USMF „Nicolae Testemițanu" în perioada anilor 2020 – 2023.
11. **Eficacitatea implementării:** Inovația propusă este importantă deoarece permite producerea de vectori lentivirali pseudotipizați cu proteina spike a virusului SARS-CoV-2, ceea ce facilitează studierea răspunsului imun la această proteină și dezvoltarea de vaccinuri și terapii împotriva COVID-19. În plus, utilizarea acestui protocol poate fi mai sigură și mai ușor de gestionat decât lucrul direct cu virusul SARS-CoV-2, ceea ce poate reduce riscurile și costurile asociate. Această inovație poate rezolva o serie de probleme asociate cu studierea și dezvoltarea de vaccinuri și terapii împotriva COVID-19, oferind o metodă mai eficientă și mai sigură de a studia comportamentul virusului, fără a fi nevoie să se lucreze direct cu virusul SARS-CoV-2.
12. **Rezultatul implementării:** Implementarea acestui protocol ar putea duce la îmbunătățirea cunoștințelor și înțelegerii despre modul în care proteina spike a virusului SARS-CoV-2 interacționează cu celulele umane și despre modul în care sistemul imun răspunde la această proteină. Acest lucru poate facilita dezvoltarea de vaccinuri și terapii împotriva COVID-19 și îmbunătăți capacitatea de a preveni și trata această boală ceea ce ar putea avea un impact semnificativ asupra sănătății publice la nivel global.
Implementarea acestui protocol ar putea, de asemenea, accelera procesul de cercetare prin simplificarea studiilor și reducerea riscurilor și costurilor implicate în manipularea virusului SARS-CoV-2. Propunerea este utilizată în practică la Disciplina de epidemiologie din cadrul Departamentului medicină Preventivă.

Prezenta inovație este implementată conform descrierii în cerere.

Șef Departamentul Cercetare,
dr. hab. șt. med., conf. univ. **Elena RAEVSCHI**

Șef Disciplina epidemiolofie
Departamentul Medicină Preventivă,
conf. univ., dr. hab. șt. med. **Angela PARASCHIV**

Coordonat: E. Groza

Anexa 6.

Act de implementare nr. 01-9/166 din 10.05.2023.
Protocol de producere a vectorilor lentivirali pseudotipizați cu proteina Spike SARS-CoV-2.

MINISTERUL SĂNĂTĂȚII AL REPUBLICII MOLDOVA
CONSILIUL MUNICIPAL CHIȘINĂU
DIRECȚIA GENERALĂ ASISTENȚĂ MEDICALĂ ȘI SOCIALĂ
IMSP Spitalul Clinic Municipal de Copii „Valentin Ignatenco"

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email: vignatenco@ms.md, www.ignatenco.md

nr. 01-9/166 din 10-05- 2023
la nr. din

APROB
Director IMSP SCMC „Valentin Ignatenco"
_____ A. HOLOSTENCO

ACT DE IMPLEMENTARE

1. **Denumirea ofertei pentru implementare: „TEST SEROLOGIC PENTRU DETECTAREA ANTICORPILOR IgG ANTI SARS-COV-2 RBD"**
2. **Autori:** ULINICI Mariana, asistent universitar.
3. **Numărul inovației:** ***Nr. 6043 din 03 mai 2023.***
4. **Unde și când a fost implementată:** Laboratorul bacteriologic IMSP SCMC „Valentin Ignatenco".
5. **Eficacitatea implementării:** Inovația propusă de un test serologic pentru detectarea anticorpilor RBD IgG anti-SARS-CoV-2 RBD este importantă prin capacitatea sa de a identifica infecțiile anterioare cu SARS-CoV-2 și răspunsurile imune provocate de vaccinurile COVID-19. Utilizarea unui epitop extrem de precis și imunogen pentru producerea de anticorpi împotriva SARS-CoV-2, cum ar fi segmentul RBD (receptor-binding domain) al proteinei spike, sporește sensibilitatea și specificitatea testului. Dezvoltarea unui test ELISA intern pentru COVID-19 poate să îmbunătățească accesibilitatea testelor, să permită personalizarea pentru a răspunde cerințelor unice ale unui anumit laborator sau ale unei cohorte de pacienți, să ofere eficiență din punct de vedere al costurilor și să faciliteze un control îmbunătățit al calității și o monitorizare continuă a eficacității testului.
6. **Rezultatele:** Inovația propusă este un test serologic pentru detectarea anticorpilor anti-SARS-CoV-2 RBD IgG reprezintă un progres semnificativ în domeniul serologiei COVID-19, cu potențialul de a avea un impact asupra sănătății publice și asupra eforturilor globale de control al pandemiei. Capacitatea de a detecta cu exactitate infecțiile anterioare cu SARS-CoV-2 și răspunsurile imune la vaccinurile COVID-19 este crucială din mai multe motive. Aceasta poate contribui la identificarea și izolarea persoanelor infectate, la depistarea contactelor și la dezvoltarea unor strategii eficiente de tratament și vaccinare. Procedeul este adresat medicilor de profil microbiologic și este utilizat în practica de laborator în IMSP SCMC „Valentin Ignatenco".

Prezenta inovație este implementată conform descrierii în cerere.

Șef laborator bacteriologic,
IMSP SCMC „Valentin Ignatenco" _____ TALMAȚCHI Vladislav

Spitalul Clinic Municipal De Copii Valentin Ignatenco

Anexa 7.

Act de implementare nr. 01-9/166 din 10.05.2023.
Protocol de producere a vectorilor lentivirali pseudotipizați cu proteina Spike SARS-CoV-2.

MINISTERUL SĂNĂTĂȚII AL REPUBLICII MOLDOVA
CONSILIUL MUNICIPAL CHIȘINĂU
DIRECȚIA GENERALĂ ASISTENȚĂ MEDICALĂ ȘI SOCIALĂ
IMSP Spitalul Clinic Municipal de Copii „Valentin Ignatenco"

str.Grenoble 149, Chișinău, Republica Moldova, tel.+373 22 208 850, fax.+373 22 725 766,
email: vignatenco@ms.md, www.ignatenco.md

APROB
Director IMSP SCMC „Valentin Ignatenco"
_____ A. HOLOSTENCO

ACT DE IMPLEMENTARE

1. **Denumirea ofertei pentru implementare: „PROTOCOL DE PRODUCERE A VECTORILOR LENTIVIRALI PSEUDOTIPIZAȚI CU PROTEINA SPIKE SARS-COV-2".**
2. **Autori:** ULINICI Mariana, asistent universitar.
3. **Numărul inovației:** ***Nr. 6045 din 04 mai 2023.***
4. **Unde și când a fost implementată:** Laboratorul bacteriologic IMSP SCMC „Valentin Ignatenco".
5. **Eficacitatea implementării:** Inovația propusă este importantă deoarece permite producerea de vectori lentivirali pseudo tipizați cu proteina spike a virusului SARS-CoV-2, ceea ce facilitează studierea răspunsului imun la această proteină și dezvoltarea de vaccinuri și terapii împotriva COVID-19. În plus, utilizarea acestui protocol poate fi mai sigură și mai ușor de gestionat decât lucrul direct cu virusul SARS-CoV-2, ceea ce poate reduce riscurile și costurile asociate. Această inovație poate rezolva o serie de probleme asociate cu studierea și dezvoltarea de vaccinuri și terapii împotriva COVID-19, oferind o metodă mai eficientă și mai sigură de a studia comportamentul virusului, fără a fi nevoie să se lucreze direct cu virusul SARS-CoV-2.
6. **Rezultatul implementării:** Implementarea acestui protocol ar putea duce la îmbunătățirea cunoștințelor și înțelegerii despre modul în care proteina spike a virusului SARS-CoV-2 interacționează cu celulele umane și despre modul în care sistemul imun răspunde la această proteină. Acest lucru poate facilita dezvoltarea de vaccinuri și terapii împotriva COVID-19 și îmbunătăți capacitatea de a preveni și trata această boală ceea ce ar putea avea un impact semnificativ asupra sănătății publice la nivel global. Implementarea acestui protocol ar putea, de asemenea, accelera procesul de cercetare prin simplificarea studiilor și reducerea riscurilor și costurilor implicate în manipularea virusului SARS-CoV-2. Procedeul este adresat medicilor de profil microbiologic și este utilizat în practica de laborator în IMSP SCMC „Valentin Ignatenco".

Prezenta inovație este implementată conform descrierii în cerere.

Șef laborator bacteriologic,
IMSP SCMC „Valentin Ignatenco" _____ TALMAȚCHI Vladislav

INFORMATION ON THE VALORISATION OF RESEARCH RESULTS

LIST OF SCIENTIFIC PUBLICATIONS AND EVENTS
at which the results of the researches for the doctoral thesis in medical sciences with the topic **„SARS-CoV-2 molecular evolution and human immune response to infection”** were presented

- **Articles in international scientific journals:**

✓ **Articles in ISI, SCOPUS journals and other international databases:**

1. **Ulinici, M.,** Covantev, S., Wingfield-Digby, J., Beloukas, A., Mathioudakis, A.G., Corlateanu, A. Screening, Diagnostic and Prognostic Tests for COVID-19: A Comprehensive Review. In: *Life*. 2021;11(6), p. 561. ISSN: 2075-1729. https://doi.org/10.3390/life11060561, (**IF: 3,817**).
2. Heydari, Z., Peshkova, M., Gonen, Z.B., Coretchi I., Eken A., Yay A.H., Dogan M. E., Gokce N., Akalin H., Kosheleva N., Galea-Abdusa D., **Ulinici M.,** Vorojbit V., Shpichka A., Groppa S., Vosough M., Todiras M., Butnaru D., Ozkul Y. & Timashev P. EVs vs. EVs: MSCs and Tregs as a source of invisible possibilities. In: *Journal of Molecular Medicine*. 2022. https://doi.org/10.1007/s00109-022-02276-2 (**IF 5.606**).
3. **Ulinici M.,** Soñora M., Orsini E., Licastro D., Monego S. D., Todiras M., Lungu L., Groppa S., Marcello A. Genome Sequences of SARS-CoV-2 Strains from the Republic of Moldova. In: *ASM Journals, Microbiology Resource Announcements*. 2022. DOI: https://doi.org/10.1128/mra.01132-22 (**IF 0.819**).
4. **Ulinici, M.,** Suljič, A., Poggianella, M., Milan Bonotto, R., Resman Rus, K., Paraschiv, A., Bonetti, A.M., Todiras, M., Corlateanu, A., Groppa, S., Ceban, E., Petrovec, M., Marcello, A. Characterisation of the Antibody Response in Sinopharm (BBIBP-CorV) Recipients and COVID-19 Convalescent Sera from the Republic of Moldova. In: *Vaccines*. 2023; 11, 637. https://doi.org/10.3390/vaccines11030637 (**IF 7.8**).

- **Articles in accredited national scientific journals:**

✓ **articoles in category B journals**

5. Cemortan, I.,Vorojbit, V., Capcelea, S., **Ulinici, M.,** Ursu, E., Croitoru, D., Grigoriev, T. Biologia virusului SARS-CoV-2: sinteză narativă. *Revista de Ştiinţe ale Sănătăţii din Moldova.* 2020, nr. 1(23), pp. 8-16. ISSN 2345-1467.
6. **Ulinici M.,** Vorojbit V. COVID-19 – teste de neutralizare. *Sănătate publică, economie şi management în medicină.* 2020, consacrat aniversării a 75-a de la fondarea USMF "Nicolae Testemițanu", 21-23 octombrie 2020. 5(87) 2020, pp.96-100. ISSN 1729-8687.

- **Abstracts/theses submitted at national or international scientific conferences**

7. **Ulinici, M.,** Vorojbit V., COVID-19 – teste de neutralizare. In: *Abstract book – Congresul (online) consacrat aniversării a 75-a de la fondarea USMF "Nicolae Testemițanu.* 21 - 23 octombrie 2020, p.178.
8. **Ulinici M,** Licastro D, Dal Monego S, Rajasekharan S, Marcello A. Înregistrarea şi publicarea rezultatelor secvenţierii complete a genomului SARS-CoV-2 ce circulă pe teritoriul RM, în repozitoriul internațional *GISAID*, 24 august 2020. https://doi.org/10.55876/gis8.221017uv.

9. **Ulinici M,** Licastro D, Dal Monego S, Rajasekharan S, Marcello A. Înregistrarea şi publicarea rezultatelor secvenţierii complete a genomului SARS-CoV-2 ce circulă pe teritoriul RM, în baza de date *NEXTSTRAIN*, august 2020.
10. **Ulinici M,** Licastro D, Dal Monego S, Orsini E, Groppa S, Todiras M, Paraschiv A, Buzurnii L, Marcello A. Înregistrarea şi publicarea rezultatelor secvenţierii complete a genomului SARS-CoV-2 ce circulă pe teritoriul RM, în repozitoriul internaţional *GISAID*, 2 Septembrie 2021. https://doi.org/10.55876/gis8.221017uv.
11. **Ulinici, M.,** Înregistrarea şi publicarea rezultatelor secvenţierii complete a genomului SARS-CoV-2 ce circulă pe teritoriul RM, în *NCBI*. 06.12.21. PRJNA786454
12. **Ulinici M.,** Development of a flow cytometry-based method to detect neutralising antibodies in SARS-COV-2 infection. *Materialele Conferinței științifico-practice naționale „Fiecare doză de vaccin contează”.* 28 aprilie 2023 la https://journal.ohrm.bba.md/index.php/journal-ohrm-bba-md/issue/view/25.
13. **Ulinici M.,** Full genome sequence of the first SARS-COV-2 isolates detected in the republic of moldova. *One Health and Risk Management*, Supplement, VOL. 2, ISSUE 4, 2021.
14. **Ulinici M.,** Production of lentivirus particles pseudotyped with SARS-COV-2 Spike protein for neutralisation or drug antiviral activity assays. *One Health and Risk Management*, materialele conferinţei naționale cu participare internaţională: „One Health Approach - achievements and challenges”, 23-24 noiembrie 2023.
15. **Ulinici M.,** A high-content imaging-based technique for detecting neutralising antibodies in SARS-COV-2 infection. *One Health and Risk Management*, materialele conferinţei internaţionale: „Tendințe actuale și provocări în medicina preventivă”, 8-9 iunie 2023.

- **Participation with communications at scientific forums:**

✓ **international**

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- **Participări la emisiuni media consacrate științei și educației, inovării și transferului tehnologic**

27. **Ulinici M.,** Interviu ICGEB: https://www.youtube.com/watch?v=Ve_6DXFZ8sM
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- **Brevete de invenții, patente, certificate de înregistrare, materiale la saloanele de invenții**

38. **Ulinici M.,** Test serologic pentru detectarea anticorpilor IgG anti SARS-COV-2 RBD. Certificat de Inovator Nr. 6043 din 03.05.2023.
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40. **Ulinici M.,** Test serologic pentru detectarea anticorpilor IgG anti SARS-COV-2 RBD. Act de implementare nr. 73 din 05.05.2023.
41. **Ulinici M.,** Test serologic pentru detectarea anticorpilor IgG anti SARS-COV-2 RBD. Act de implementare nr. 01-4/79 din 05 mai 2023.
42. **Ulinici M.,** Test serologic pentru detectarea anticorpilor IgG anti SARS-COV-2 RBD. Act de implementare nr. 01-9/166 din 10.05.2023.
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- **Stagiu de cercetare peste hotare**

46. THE ARTURO FALASCHI ICGEB Short-term FELLOWSHIP at a PhD level (F/MDA20-01). Perioada: 04.06.21-30.08.21.
47. Visiting researcher în cardul proiectului: Capacity building in Virus Surveillance to tackle COVID-19 and beyond. Parteneri: ICGEB, Trieste Italia. (ICGEB, grant nr. CUP:D87D20000020009), Laboratorul de virusologie moleculară, ICGEB, Trieste, Italia: 15.10.21- 19.12.21

Declaraţia privind asumarea răspunderii

Subsemnata, declar pe răspundere personală, că materialele prezentate în teza de doctorat sunt rezultatul propriilor cercetări şi realizări ştiinţifice. Conştientizez că, în caz contrar, urmează să suport consecinţele în conformitate cu legislaţia în vigoare.

Ulinici, Mariana

Semnătura

Data

Declaration on accountability

I declare the personal responsibility that information presented in this thesis are the result of my own research and scientific achievements. I realize that, otherwise, will suffer the consequences in accordance with law.

Ulinici, Mariana

Signature

Date

Déclaration sur la responsabilité

Je déclare la responsabilité personnelle que les informations présentées dans cette thèse sont le résultat de mes propres recherches et réalisations scientifiques. Je me rends compte que, sinon, en subiront les conséquences conformément à la loi.

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Signature

Date