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Comparative Analysis of High-Performance Bioanalytical Methods for SARS-CoV-2 Serodiagnosis

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ABSTRACT

The SARS-CoV-2 pandemic was declared a global health emergency by the World Health Organization in March 2020 and represented one of the most significant biomedical challenges of the 21st century. The unprecedent scale of viral transmission, clinical heterogeneity, and global impact on healthcare systems underscored the urgent need for rapid, sensitive, and reliable diagnostic tools. In addition to molecular testing, which played a central role in the identification of active infections, serological assays became essential for assessing the magnitude, duration, and quality of immune responses following both natural infection and vaccination.

This study presents a comparative analysis of three high-performance serological platforms: Enzyme-Linked Immunosorbent Assay (ELISA), Luminex, and Meso Scale Discovery (MSD). —The aim was to quantify specific antibodies against the major SARS-CoV-2 antigens, corresponding to the full-length Spike protein (S), the receptor-binding domain (RBD), and the Nucleocapsid (N) protein. Fifty human serum samples from PCR-confirmed SARS-CoV-2-positive donors were analyzed to evaluate each platform's analytical performance, including sensitivity, reproducibility, dynamic range, and multiplexing capability.

Correlation analyses demonstrated a high degree of concordance among the three systems, with correlation coefficients exceeding 0.9 for S and RBD antigens, confirming the strong reliability and inter-assay agreement of quantitative antibody detection. Furthermore, integrating IgG avidity assessment within the multiplex framework provided qualitative insight into antibody maturation, revealing that vaccination or booster doses induce antibodies with higher avidity and enhance neutralizing potential compared to those generated by natural infection.

The results confirm that multiplex immunoassays such as Luminex and MSD offer superior analytical power, efficiency, and data richness compared to conventional ELISA, enabling comprehensive seroprofiling from minimal sample volumes. These technologies represent a valuable asset for immunological surveillance and vaccine monitoring, paving the way for an integrated and high-resolution approach to immunodiagnostics and global health preparedness.

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ABBREVIATION LIST

+ssRNA	Positive-Sense Single-Strand RNA
ACE2	Angiotensin-Converting Enzyme 2
Ads	Adenovirus
APC	Antigen Presenting Cell
arcRNA	Antigen-Encoding Replicon RNA
ARDS	Acute Respiratory Distress Syndrome
AU	Arbitrary Units
BAU	Binding Antibody Units
CBER	Center for Biologics Evaluation and Research
CDER	Center for Drug Evaluation and Research
circRNA	circular mRNA
CNS	Central Nervous System
COVID- ETF	COVID- 19 EMA Pandemic Task Force
COVID-19	Coronavirus Disease 2019
CTAP	Coronavirus Treatment Acceleration Program
CV	Coefficient of Variation
E	Envelope
ECL	Electrochemiluminescence
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
ETFM	Emergency Task Force Mechanism
EUA	Emergency Use Authorization
FCS	Furin Cleavage Site
FDA	Food and Drug Administration
FP	Fusion Peptide
HBsAg	Hepatitis B Surface Antigen
HCl	Hydrochloric acid
HCV	Hepatitis C Virus
HHS	Health and Human Services
HIV	Human Immunodeficiency Virus
HR	Heptad Repeat

LNPs	Lipid Nanoparticles
M	Membrane
mAbs	Monoclonal Antibodies
MAC	Membrane Attack Complex
MERS- CoV	Middle East Respiratory Syndrome Corona
MHC	Major Histocompatibility Complex
MSD	Meso Scale Discovery
N	Nucleocapsid
NFDM	Non-fat Dairy Milk
NGS	Next Generation Sequencing
NK	Natural Killer
Nsp	Non-Structural Protein
NTD	N-Terminal Domain
OD	Optical Density
ORF	Open Reading Frame
PASC	Post Acute *Sequaele* of SARS- CoV-2
PCR	Polymerase Chain Reaction
PD	Peptidase Domain
PHEIC	public health emergency of international concern
RAAS	Renin-Angiotensin-Aldosterone System
RBD	Receptor Binding Domain
RBM	Receptor Binding Motif
RLU	Relative Light Unit
RNA	Ribonucleic Acid
RT- PCR	Reverse Transcript- Polymerase Chain Reaction
S	Spike
saRNA	Self-Amplifying RNA
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
sIgA	Secretory IgA
TBS	Tris Buffered Saline
TMB	3,3’,5,5’-Tetramethylbenzidine
UTRs	untranslated regions
VoC	Variant of Concern

VoI	Variant of Interest
WHO	World Health Organization

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1 INTRODUCTION

SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) is a newly identified coronavirus that emerged in late 2019, distinguished by its high pathogenic potential and rapid transmissibility (To KK. et al., 2021). This virus is responsible for COVID-19 (Coronavirus Disease 2019), an acute respiratory syndrome that has triggered a global health crisis. Beyond its clinical implications, the pandemic has profoundly affected societies and economies worldwide, reshaping public health priorities, disrupting daily life, and challenging healthcare systems on an unprecedented scale (Pak A., 2020).

Phylogenetic analyses suggest that SARS-CoV-2 virus belongs the genus *Betacoronavirus*, (Gao T. et al., 2021). This taxonomic classification places it in the same group as other human-pathogenic coronaviruses, such as SARS-CoV-1 and MERS-CoV (Middle East Respiratory Syndrome Corona Virus), which have been associated with severe acute respiratory syndromes characterized by high morbidity and mortality (Huang C et al., 2020; V'kovski P. et al., 2021; Gao T. et al., 2021). Human coronaviruses are known to cause a broad spectrum of respiratory conditions, ranging from mild, self-limiting infections, typically associated with seasonal strains such as HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1, to more severe illnesses, particularly in vulnerable populations such as the elderly or individuals with underlying health conditions (Firouzabadi N. et al.,2023).

In this context, the emergence of SARS-CoV(2002), MERS-CoV (2012), and SARS-CoV-2 (2019) marked a significant shift in pathogenic potential. These viruses demonstrated markedly higher virulence, often leading to serious clinical manifestations, including Acute Respiratory Distress Syndrome (ARDS), and contributed to substantial public health challenges due to their epidemic and pandemic spread. (Boechat J.L. et al., 2021; V'kovski P. et al., 2021). From the very beginning of the pandemic, SARS-CoV-2 infection posed considerable diagnostic and clinical challenges, primarily due to its broad spectrum of manifestations. These ranged from asymptomatic or mild presentations to severe cases involving acute respiratory failure and multi-organ dysfunction (Illi B. et al., 2020; Mazhari R. et al., 2021). The clinical overlap with symptoms caused by other seasonal respiratory viruses circulating in Italy further complicated differential diagnosis, especially in the absence of specific and widely available diagnostic tools (Bordi L. et al., 2020).

In the early stages of the outbreak, scientific understanding of the novel virus was still limited. However, airborne transmission was already strongly suspected, and subsequent evidence confirmed that COVID-19 spreads predominantly through three main routes (Morawska L. & Milton D. K., 2020):

- Direct contact with the mucous membranes of the eyes, nose, or mouth of infected individuals;
- Indirect contact via contaminated surfaces;
- Aerosol transmission, through respiratory droplets generated by speaking, coughing, or sneezing—droplets that can remain suspended in enclosed environments for up to three hours (Chung Y.S. et al., 2024).

This complex transmission dynamic, combined with the virus's variable clinical presentation, underscored the urgent need for robust surveillance systems and targeted public health interventions.

To contain its rapid spread, governments around the world implemented restrictive measures aimed at reducing individual and collective mobility. In Italy, on March 9th, 2020, a nationwide lockdown was imposed, suspending all non-essential activities and limiting movement to work, health-related needs, or other essential reasons (Bezzini D. et al, 2021). Indeed, among the earliest non-pharmaceutical interventions were physical distancing and hand hygiene, both fundamental in reducing virus transmission via droplets. (Rohit A. et al., 2020; Paul S. et al., 2024).

The use of face masks also played a pivotal role in mitigating transmission (Morawska L. & Milton D.K., 2020).

During the initial phase of the pandemic, containment strategies primarily centred on the early identification of cases, the prompt isolation of infected individuals, and, where isolation was not feasible, the implementation of targeted measures to reduce the risk of viral transmission in shared environments. These efforts were crucial in limiting the spread of SARS-CoV-2, especially in the absence of effective therapeutic options. Simultaneously, the unprecedented pace of vaccine development and subsequent regulatory approval of SARS-CoV-2-specific vaccines enabled a significant shift in public health strategy. Mass vaccination campaigns were launched globally with the ambitious objective of immunizing at least 70% of the world's population, thereby reinforcing containment efforts and paving the way toward long-term pandemic control (Doshi R. H. et al., 2024).

1.1 EPIDEMIOLOGY

The SARS-CoV-2 virus was declared a pandemic on 11th March 2020 by the World Health Organization (WHO) and marked a turning point in global public health, reshaping priorities in diagnostics, prevention, and immunological surveillance (Cucinotta D. et al., 2020). A pandemic is defined as the worldwide spread of a new disease that affects at least two continents, with sustained human-to-human transmission, regardless the severity of the illness (Kelly H. et al., 2011).

Emerging and re-emerging pathogens posed a serious challenge to public health, and the COVID-19 pandemic clearly demonstrated the need for a coordinated, interdisciplinary international response (Zhu N. et al., 2019).

1.1.1 Origin of COVID-19

Coronaviruses exhibit a broad host tropism, enabling them to infect not only humans but also a wide range of animal species (Hu B. et al., 2021). This capacity to cross species barriers results in different clinical manifestations depending on the host organism. Such biological versatility supports the widely accepted hypothesis of zoonotic spillover, particularly from bats, which is believed to have facilitated the initial transmission of SARS-CoV-2 to humans. The virus's rapid spread within a population lacking prior immunity, underscores the critical role of host tropism in shaping both the emergence and the epidemiological trajectory of novel coronaviruses (V'kovski, Philip et al., 2021; Hurt A.C. et al., 2021; Carabelli A.M. et al., 2023). Pandemics caused by MERS, SARS-CoV, and SARS-CoV-2, originated in Asian regions, where local dietary practices include the consumption of wild animals, often sold in so-called "wet markets." These markets are characterized by the sale of seafood, fish, crustaceans, carcasses, and a wide variety of live wild animals such as hedgehogs, badgers, snakes, and birds (Wu F et al., 2020). Many of these animals either feed on bats or share habitats with them, increasing the likelihood of interspecies viral transmission. Given that bats are well-established natural reservoirs for numerous viruses, including coronaviruses (Zhou P et al., 2020; El-Sayed and Kamel, 2021). It is therefore hypothesized that the virus was transmitted from bats to humans through an intermediate host (Hu B. et al., 2021) (*Figure 1*).

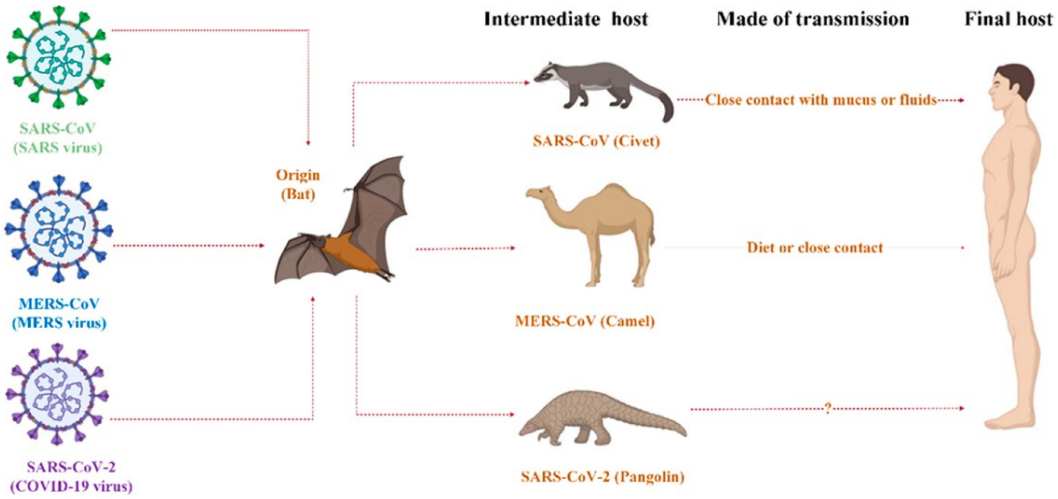

Figure 1. Coronaviruses zoonotic spillover *(Salahshoori I et al., 2021)*

The zoonotic spillover theory of SARS-CoV-2 is based on high genetic similarity between the virus isolated from the first COVID-19 patients and coronaviruses found in bats. In these animals, mild respiratory manifestations are found. (Gupta S.K. et al., 2022). This phenomenon occurs more frequently in rural areas, where deforestation and urbanization increase the risk of spillover (Lytras S. et al., 2021). An emerging phenomenon of growing interest is the "reverse spillover" or "spillback," which refers to the possibility that, after adapting to humans, the virus is transmitted back to susceptible animals (Olival KJ.et al., 2020). This process can promote the emergence of new viral variants through mutations accumulated in non-human hosts, generating a reverse evolutionary cycle with potential implications for public health, zoonosis management, and vaccine strategies. Spillback of SARS-CoV-2 has already been documented in several animal species, including primates and other mammals. New variants developed in these hosts may then be retransmitted to humans, creating a continuous cycle of viral adaptation (Milich K.M. et al., 2024).

1.1.2 From Local Outbreak to Global Pandemic

The first alerts of COVID 19 pandemic were issued in December 2019, when cases of pneumonia of unknown origin were reported in Wuhan, Hubei Province (China) where 27 patients were hospitalized with a severe form of viral pneumonia, associated with fever, cough, chest pain, and, in the most severe cases, dyspnoea and lymphocytic interstitial pneumonia (Huang C. et al., 2020; Hu B. et al., 2021). All early cases were epidemiologically linked to the Huanan seafood market, which was subsequently closed on January 1st, 2020. The first death in China caused by SARS-CoV-2 was reported on

January, 2020, followed by the detection of the first infection case in Thailand. The high transmissibility of the virus, combined with the large number of international flights, led to a sharp and rapid increase in cases worldwide, at a rate higher than that observed during the SARS-CoV pandemic (Wu JT. et al., 2020).

This situation raised serious concerns for global health, and with the aim to limit the spread to other countries, the first travel restrictions to and from China were implemented (Meng X. et al., 2023). However, these measures proved to be insufficient, and less than a month later, clusters of infected patients were reported in more than 29 countries, including several in Europe (Park M et al., 2020).

What initially appeared to be a localized outbreak, rapidly evolved into a global health crisis, facilitated by the high international mobility and the absence of pre-existing immunity in the population (Huang C. et al., 2020; Boechat J.L. et al., 2021). On January 30th, 2020, the WHO officially declared the COVID-19 outbreak a public health emergency of international concern (PHEIC) and subsequently a pandemic on March 11th, 2020 (Jee Y, 2020) *(Figure 2).*

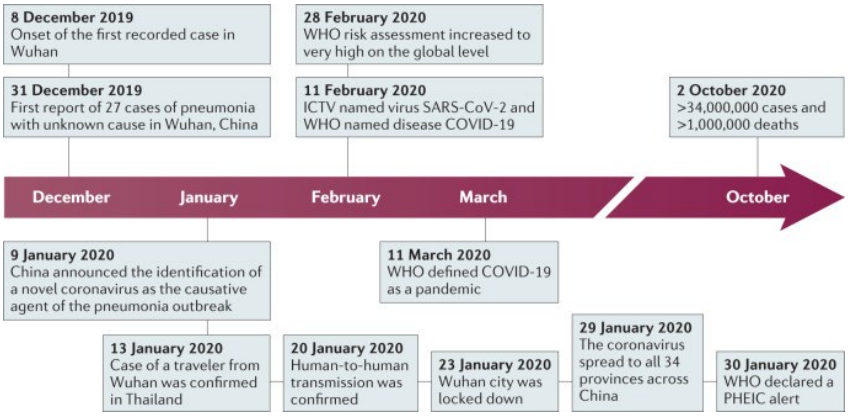

Figure 2. Chronological summary of significant events during the COVID-19 outbreak *(Hu B. et al., 2021)*

The epidemiology of SARS-CoV-2 has undergone rapid changes, influenced by several factors: the emergence of new viral variants, the immune response of the population, the effectiveness of containment measures, and—most notably—the widespread introduction of vaccines. In the early months of the pandemic, SARS-CoV-2 exhibited transmission dynamics similar to those of other respiratory pathogens. However, as the pandemic progressed, more transmissible variants (e.g., Alpha/B.1.1.7) emerged, leading to higher effective reproduction numbers and faster, broader dissemination of the virus (Sanche S. et al. 2020; Achaiah N.C. et al.,2020; Galloway S.E. et al. 2021).

The early waves of the COVID-19 pandemic were marked by elevated rates of hospitalization and mortality, particularly among elderly individuals and those with underlying health conditions. As vaccination coverage expanded and natural immunity began to develop within the population, clinical outcomes gradually improved, accompanied by a significant reduction in lethality (Dantas Filho F.F et al., 2023)

Unlike other respiratory viruses such as influenza, which typically follow seasonal patterns with peaks during the winter months, SARS-CoV-2 demonstrated a remarkable ability to transmit across diverse climatic conditions Nevertheless, epidemiological data consistently show an increase in incidence during colder periods. This seasonal trend is likely influenced by behavioral factors—such as increased time spent indoors—and environmental conditions that favor viral persistence and transmission (Wiemken TL. et al., 2023).

On a global scale, 2021 recorded the highest number of COVID-19-related deaths, underscoring the devastating impact of the pandemic prior to the widespread availability and uptake of vaccines (*Figure 3*).

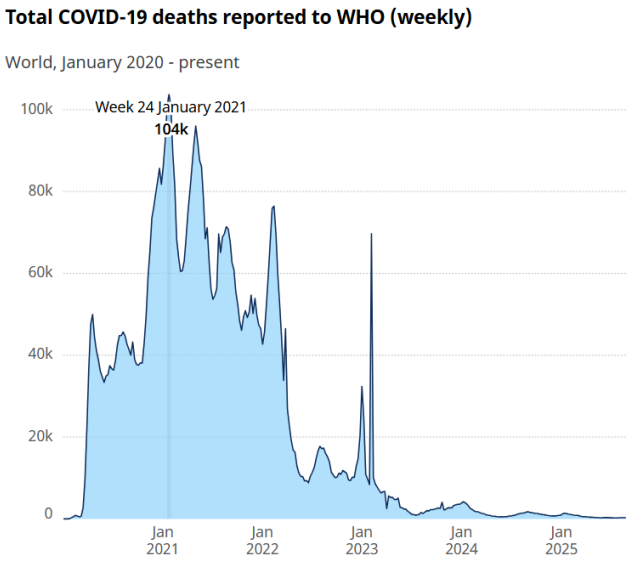

Figure 3. Globally weekly total COVID- 19 deaths. This *graph illustrates the weekly total COVID-19 deaths reported globally to the WHO from January 2020 to the present year. The vertical axis represents the number of deaths per week, while the horizontal axis spans time from January 2020 through early 2025. The highest weekly death toll occurred around the 24th week of January 2021, reaching approximately 104,000 deaths. Data were obtained and adapted from the World Health Organization- COVID-19 Deaths Dashboard.*

By 2025, the trajectory of the pandemic has taken on endemic characteristics, with a significant decline in both incidence and lethality. According to the most recent WHO

data, between January 6th and February 2nd, 2025, approximately 147,000 new cases and 4,500 deaths were reported worldwide, with a daily average of about 160 deaths (World Health Organization. COVID-19 Deaths Dashboard.). This suggests an improved control of viral spread and greater population protection through acquired immunity.

1.2 VIROLOGY

SARS-CoV-2 belongs the order *Nidovirales* and the family *Coronaviridae*, which includes two subfamilies: *Letovirinae* and *Orthocoronavirinae*. The latter comprises the genera *Alphacoronavirus, Gammacoronavirus, and Deltacoronavirus,* as well as the genus *Betacoronavirus,* which includes SARS-CoV-2 (Yao H. et al. 2020) *(Figure 4).*

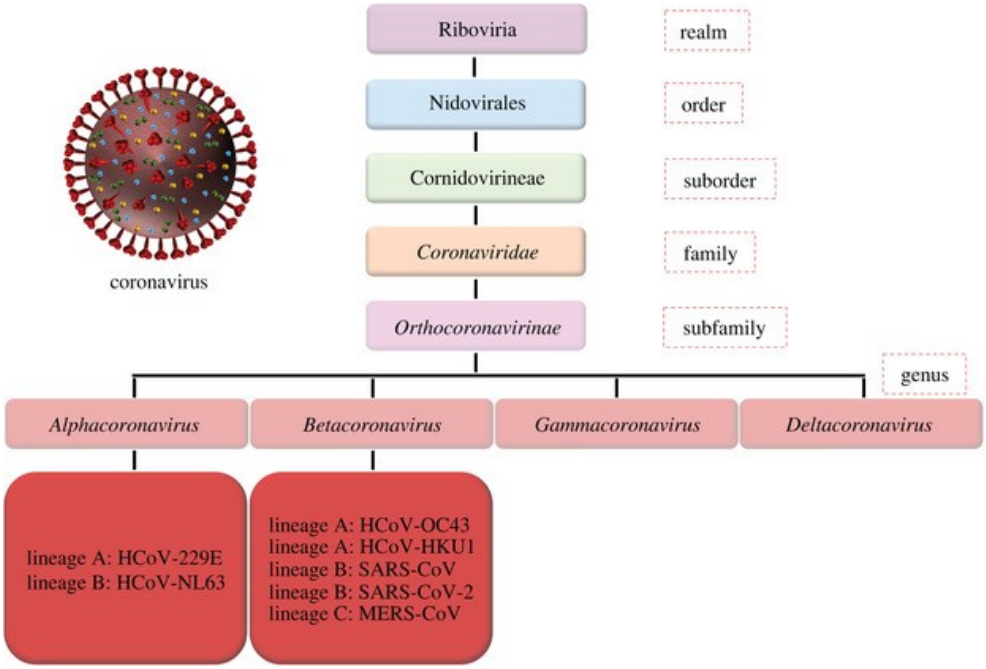

Figure 4 Classification of Coronaviruses *(Aydogdu, M. O et al., 2021)*

The first coronavirus, HCoV-229E, was discovered in 1966, followed by the identification of six other species: HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV, and SARS-CoV-2 (Tang G. et al., 2022).

1.2.1 Molecular Structure and Genome Organization of SARS-CoV-2

Coronaviruses have a diameter ranging from 60 to 140 nm, as revealed by electron microscopy techniques (Tang D. el a., 2020). The virus is enclosed by a pericapsid, a lipid bilayer derived from the host cell membrane, on which trimers of the spike (S) protein are anchored, resembling the crown-like spikes that give the virus its name.

The main structure of the virus consists of four proteins: membrane protein (M), envelope protein (E), spike protein (S), and nucleocapsid protein (N). All structural proteins are embedded in the viral envelope, except for the N protein, which directly interacts with the RNA located in the central part of the viral particle, thus forming the nucleocapsid (Ashour HM et al., 2020; Malik Y.A, 2020) (*Figure 5*).

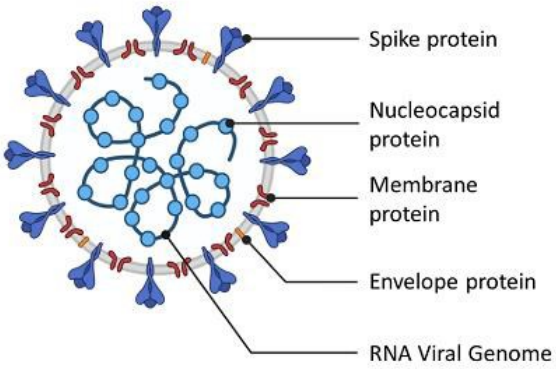

Figure 5 SARS-CoV-2 structure *(Nayak S.S et al., 2023)*

The N protein plays a crucial role in supporting genome replication and assembly of new virions. Additionally, it interferes with the host immune response (McBride R et al., 2014; Gao T. et al., 2021).

The S protein is a surface class I fusion homotrimeric glycoprotein with an approximate molecular weight of 600 kDa, which enables viral entry by binding to the Angiotensin converting enzyme 2 (ACE2) receptor on host cells and facilitates membrane fusion (Huang Y et al., 2020). Each monomer consists of two functional subunits: S1, which contains the N-terminal domain (NTD) and the Receptor-Binding Domain (RBD), and S2, which mediates membrane fusion and includes two heptad-repeat regions (HR-N and HR-C) (Yao H. et al., 2020; Hurt AC et al., 2021; Kumar A. et al., 2021). Within the RBD, a structural core and a receptor-binding motif (RBM) can be distinguished, the latter being directly involved in ACE2 recognition (Shang J. et al., 2020). The ACE2 is widely expressed in a variety of human tissues, influencing viral tropism and contributing to the broad clinical symptoms' spectrum observed with COVID-19 (V'kovski P. et al., 2021).

The M protein (25-30 KDa) is the most abundant component in viral particles and plays and essential role in maintaining the virion shape and orchestrating the assembly of viral components into mature virions (Neuman B.W. et al., 2006).

Lastly, the E protein (8-12 KDa) is critical for virus budding and release, as well as contributing to the pathogenicity of SARS-CoV-2 by causing cellular damage (Schoeman D. & Fielding B.C., 2019).

SARS-CoV-2 is a positive-sense single-strand RNA (+ssRNA) virus, which harbours the largest genome known among RNA viruses, ranging from 27 to 32 kilobases (Yao H. et al., 2020; Kumar A. et al., 2021). The genetic sequence of wild-type SARS-CoV-2 consisting of 29,881 nucleotides was first released on January 12th, 2020 (Yao H. et al., 2020; Kumar A. et al., 2021; Fang E. et al., 2022). The +ssRNA comprise six major Open Reading Frames (ORFs), which encode 29 proteins, including sixteen non-structural proteins (nsp1–nsp16), encoded within the ORF1a/ORF1b region and the four structural proteins (Yao H. et al., 2020; Carabelli A.M. et al., 2023) *(Figure 6)*.

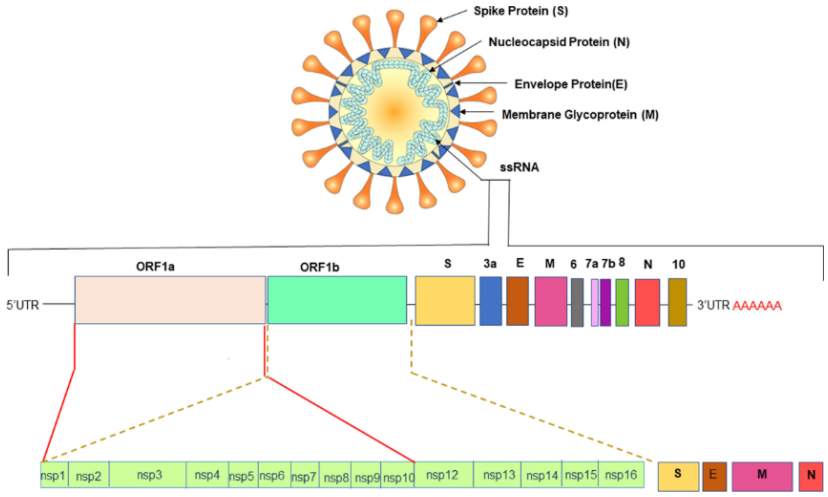

Figure 6 SARS-CoV-2 genome. *Schematic representation of the SARS-CoV-2 genome (~29.9 kb, +ssRNA). The genome includes 5'and 3'untranslated regions (UTRs), which regulate replication and translation. The ORF1a/ORF1b region encodes 16 non-structural proteins (nsp1–nsp16) involved in replication. The 3'end of the genome contains structural protein genes (S, M, E, N) and several accessory ORFs (e.g., ORF3a, ORF6, ORF7a/b, ORF8) (Akter N et al., 2021)*

1.2.2 SARS-CoV-2 Replication Mechanisms

SARS-CoV-2 replication begins with the entry of the virus into the host cell via endocytosis, following the binding of the RBD the ACE2 receptor (Sanaie S. et al., 2021; Nazir F. et al., 2024;). Subsequently, the S2 subunit mediates the fusion of viral and cellular membranes, enabling the release of the viral genome into the cytoplasm. A key factor in viral entry is the presence of a furin cleavage site (FCS) at the junction between the S1 and S2 subunits, its proteolysis facilitates membrane fusion and viral entry. Mutations enhancing FCS activation have been linked to increased viral fitness and transmissibility, as observed in the Alpha and Delta variants, which demonstrated a ~60% higher transmissibility compared to earlier strains (Carabelli A.M. et al., 2023).

Inside the host cell, the ORF1a and ORF1b regions of the viral RNA are translated into two polyproteins, which are subsequently cleaved into 16 non-structural proteins (Nsp)

(Nazir F. et al., 2024). Translation of ORF1b occurs via a ribosomal frameshifting mechanism, allowing the production of a single extended polyprotein (Willet BJ et al., 2022).

Following ORF1a/b transcription, a specialized microenvironment is created for viral replication (Kumar A. et al., 2020). Structural proteins such as E and N localize themselves into the endoplasmic reticulum and in the final phase of the replication cycle, M and E proteins contribute to the assembly of complete virions. These newly formed virions are carried toward the plasma membrane *via* vesicles, which fuse themselves with the membrane and release the virions through exocytosis (Katiyar H. et al, 2024) *(Figure 7)*.

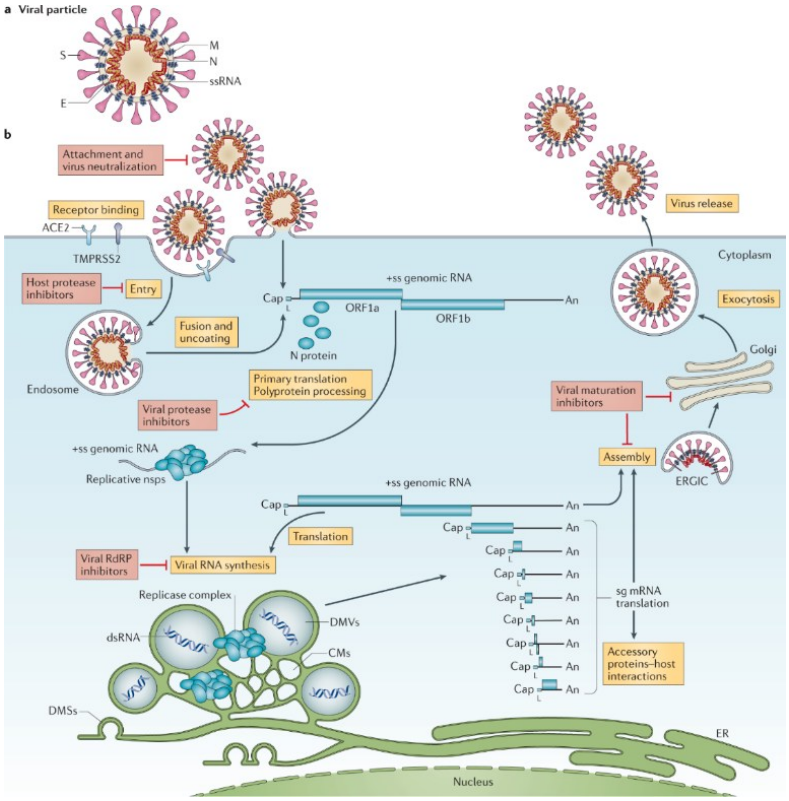

Figure 7. SARS-CoV-2 replication cycle *The image illustrates the main stages of the SARS-CoV-2 replication cycle. The virus binds to the ACE2 receptor via the S protein and enters into the host cell through endocytosis. Once in the cytoplasm, the single +ssRNA is translated into nsps, including RdRp, which is essential for viral replication. Following the formation of the replicase-transcriptase complex, subgenomic RNAs and structural proteins (S, M, E, and N) are synthesized. Assembly of new virions takes place within vesicles derived from the endoplasmic reticulum and Golgi apparatus, from which they are carryed to the cell membrane and released via exocytosis. (V'kovski P et al., 2021)*

During replication and protein translation, mutations that alter viral proteins sequence and consequently potentially impair recognition by pre-existing antibodies, may arise. Mutations in the RBD of the S protein are particularly significant, as they can modify binding affinity to ACE2 and reduce the efficacy of neutralizing antibodies (Xue S. et al., 2024). This may contribute to lower vaccine effectiveness and reinfections (Yuki K. et al., 2020; Sanaie S. et al., 2021).

Based on parameters such as transmissibility, clinical severity, immune escape, and impact on diagnostics or therapeutics, the World Health Organization (WHO) categorizes certain mutations as Variants of Concern (VOC) or Variants of Interest (VOI) (Carabelli A.M. et al. 2023; WHO 2023 (a)).

1.3 PATHOGENESIS

The clinical severity of SARS-CoV-2 infection depends on multiple factors, including viral load, patient age, co-morbidities, and pre-existing immune status (Mazhari R. et al., 2021; Quinti I. et al., 2021). The infection manifests with a wide spectrum of symptoms, ranging from mild and self-limiting to severe and sometimes fatal forms (*Figure 8*) (Magro C. et al., 2021).

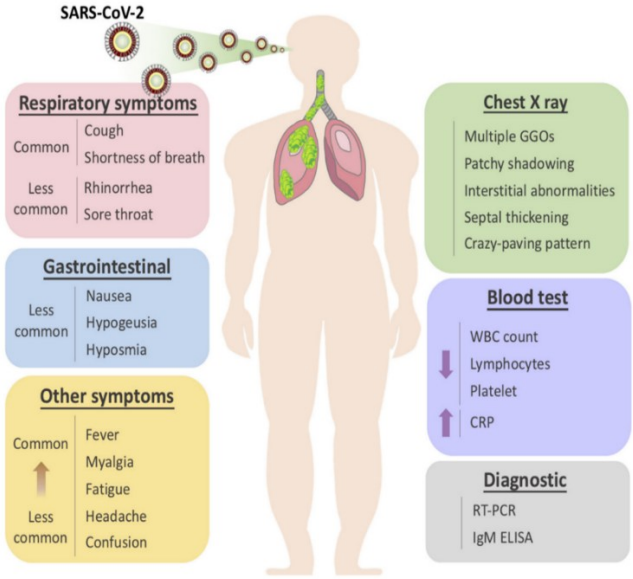

Figure 8 Most common COVID-19 symptoms *(Tu Y.F et al., 2020)*

Following infection, the average incubation period of SARS-CoV-2 is 4–5 days before symptom onset. In most cases (97.5%), symptomatic individuals develop the disease within 11.5 days (Lauer SA. et al., 2020). The main symptoms include dry cough and fever, which can progress to shortness of breath, muscle and joint pain, severe headache, persistent diarrhea, and hemoptysis andhospitalization (Shang J. et al., 2020). Around 5–6 days after the appearance of symptoms, SARS-CoV-2 reaches its peak replication and infectivity, while after 8–9 days, in the most severe cases, acute respiratory distress syndrome may develop potentially progressing to pulmonary fibrosis and requiring

mechanical ventilation. This condition is characterized by severe breathing difficulties and low blood oxygen levels, making patients more susceptible to bacterial and fungal co-infections (Tay M.Z. et al., 2020; Buehler PK et al., 2021)

1.4 CLINICAL MANIFESTATIONS

ACE2 is highly expressed in type II alveolar cells, which are essential for maintaining alveolar integrity (Silva MG. et al., 2022). Infection of these cells triggers the innate immune response, causing widespread inflammation, interstitial and intra-alveolar edema, increased surface tension, alveolar collapse, and hypoxemia (Wang LL. et al., 2022). Although ACE2 inhibition was initially considered as a potential strategy to mitigate ARDS, experimental studies suggest that its absence may lead to adverse effects such as hemodynamic dysfunction and cardiovascular complications (Usman AM et al., 2021).

High ACE2 expression in the olfactory epithelium explains early anosmia (Chen M et al., 2020), and its presence in over 70 human tissues accounts for systemic involvement, including gastrointestinal, cardiovascular, and nervous systems (Hamming I. et al., 2004; Friedel DM et al., 2023). Under physiological conditions, ACE2 is a key regulator of the renin–angiotensin–aldosterone system (RAAS), contributing to cardiovascular, respiratory, and metabolic homeostasis (Usman A. M. et al., 2021). Endocytosis of the virus leads to downregulation of ACE2 on the cell surface, coupled with increased serum levels of angiotensin II, and pro-inflammatory responses, contributing to ARDS (Sanaie S. et al., 2021).

Systemic manifestations include:

- Cardiovascular: Downregulation of ACE2 promotes vasoconstriction and endothelial inflammation (Abrignani MG et al., 2022).
- Gastrointestinal: ACE2 in glandular cells underlies nausea, vomiting, diarrhea, and abdominal pain (Gupta A. et al., 2020).
- Nervous system: Central Nervous System (CNS) entry via the olfactory bulb and other ACE2-rich regions may cause headache, cognitive impairment, anosmia, and dysgeusia (Sayin I et al., 2020).
- Skin: Reported manifestations include vesicles, morbilliform rashes, and chilblains (“COVID toes”) (Magro C et al., 2021) (*Figure 9).*

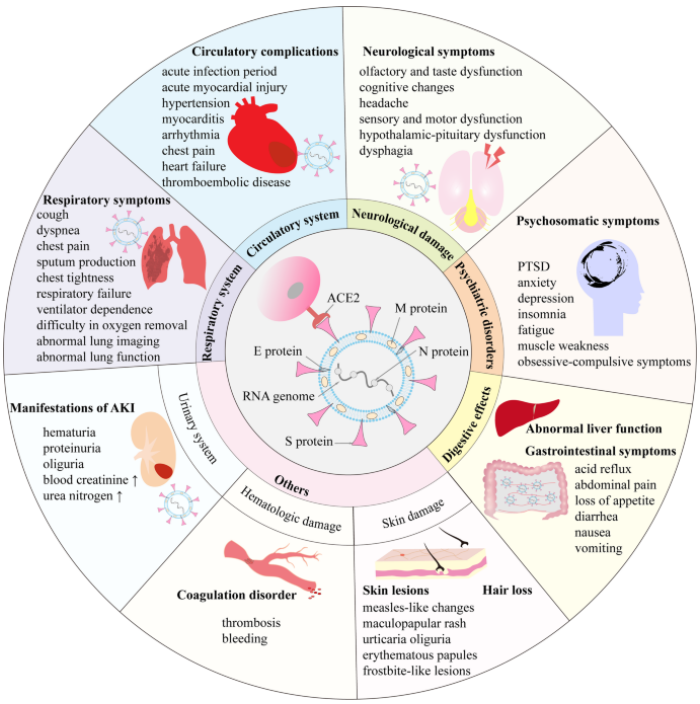

Figure 9. Systemic and multi-organ manifestations associated with SARS-CoV-2 infection*. The image illustrates the main organs and systems potentially affected during both the acute and post-acute phases of COVID-19. Beyond the respiratory system, SARS-CoV-2 can cause systemic effects involving the cardiovascular system (myocarditis, thrombosis), central nervous system (headache, confusion, anosmia), gastrointestinal tract (diarrhoea, nausea), renal and endocrine systems, as well as musculoskeletal symptoms (myalgia, fatigue). These manifestations highlight the multisystemic nature of the disease and the key role of the immune-inflammatory response (Jiao T et al., 2024)*

Several years after the onset of the SARS-CoV-2 pandemic, millions of individuals worldwide continue to report persistent symptoms associated with a clinical condition known as Long COVID. Originally coined by the general public, the term has since been adopted by international health authorities. Also referred to as Post-Acute *Sequelae* of SARS-CoV-2 infection (PASC), this syndrome encompasses a heterogeneous and multisystemic symptom profile (Rolf M., 2023; Liu et al., 2023).

According to the WHO, Long COVID is defined as "*the persistence or onset of new symptoms three months after the initial infection with SARS-CoV-2, lasting for at least two months, and not explained by an alternative diagnosis*" (Greenhalgh T. et al., 2024). The syndrome can affect individuals of any age or sex, including those who were previously healthy. However, it is more frequently observed in people with comorbidities, obesity, those who were unvaccinated, or those who experienced severe forms of COVID-19 (Nittas V. et al., 2022).

Clinically, Long COVID is characterized by a highly variable psychological and multisystemic symptomatology. To date, over 200 symptoms potentially attributable to the condition have been identified but most common include chronic fatigue and

persistent musculoskeletal pain (Liu Y. et al., 2023; Greenhalgh T. et al., 2024). Respiratory and gastrointestinal symptoms are also frequent as well as neuropsychological manifestations such as anxiety, depression, and memory problems, often impairing patients' ability to return to their pre-infection daily routines and work activities (CDC, 2025) (*Figure 10*).

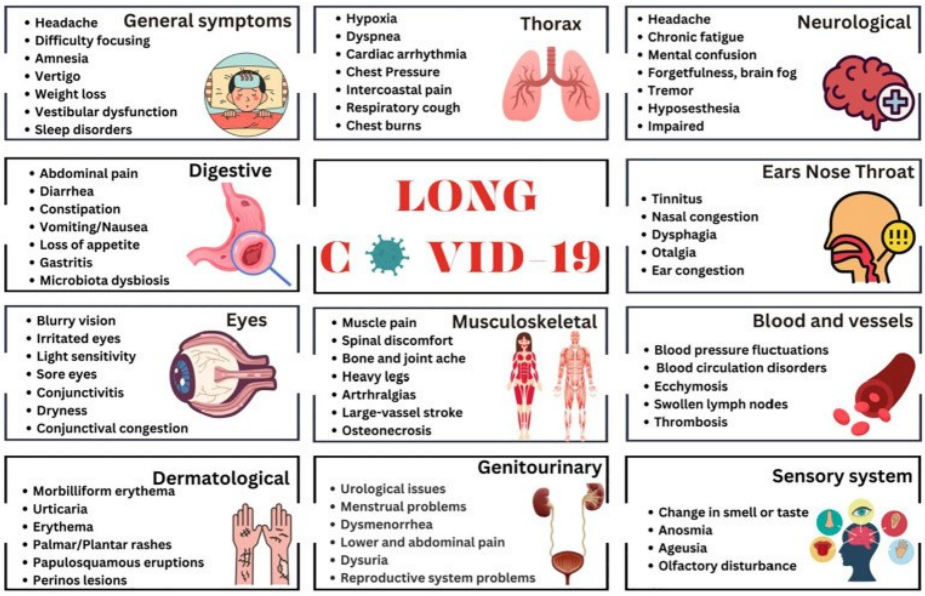

Figure 10. Main symptoms of Long COVID *The image illustrates the broad heterogeneity and systemic distribution of symptoms reported by patients with post-COVID-19 syndrome (Gheorghita R. et al., 2024)*

The pathophysiological mechanisms underlying Long COVID are not yet fully understood. Leading hypotheses include pre-existing or infection-induced autoimmunity, viral persistence in a latent form, and reactivation of dormant pathogens (Peluso M.J.. et al., 2022; Liu Y. et al., 2023)

1.5 IMMUNOLOGICAL MECHANISM IN COVID-19

The first barrier against all the pathogens is represented by innate immunity, a rapid and non-specific system that plays a crucial role in the early containment of the infection. The effectiveness of this response determines not only the individual clinical outcome but also the virus's ability to spread within the population (Carabelli AM. et al., 2023).

A prompt and well-coordinated innate response, characterized by early activation of type I interferons and effector cells, can limit viral replication and reduce the risk of systemic inflammatory responses. However, if this response is not properly regulated, it can lead to systemic inflammation and tissue damage. In particular, high levels of IL-1 and IL-6 have been associated with severe forms of COVID-19 and increased mortality (Sanaie S et al., 2021; Netea MG et al., 2023).

Natural Killer (NK) cells, an integral part of innate immunity, play a key role not only in the direct lysis of infected cells but also in modulating the adaptive immune response through interactions with dendritic cells. In COVID-19 patients, NK cell dysfunction has been observed, linked to increased inflammatory cytokines that suppress their cytotoxic function. This phenomenon may promote viral spread, exacerbate inflammation, and contribute to tissue damage, particularly in the lungs (Masselli E. et al., 2020).

Therefore, an imbalance in the innate response not only compromises the initial control of the infection but can also hinder the effective activation of adaptive immunity, with significant clinical consequences (Wong LR. & Perlman S., 2022). IgA antibodies represent the primary line of defence at the mucosal level, especially in the respiratory tract, the main entry point for the virus. There are two forms: IgA1, predominant in the airways, and IgA2, more common in the intestine. Secretory IgA (sIgA), also present in saliva and breast milk, providing both local and systemic protection (Quinti I et al.,2021). Serum IgA levels tend to decline within a month after symptom onset, whereas mucosal IgA can persist for longer. According to Sterlin D. et al. (2021), IgA antibodies appear within three weeks and often provide better protection than IgG (Sterlin D. et al., 2021). Considering that human mucosae cover a surface area of over 400 m², their role as an immune barrier is essential (Zhou X. et al., 2025).

1.5.1 Serological Dynamics and Anomalies in the Antibody Response to SARS-CoV-2

In most infections, the antibody response begins after symptom onset and the peak of viral replication, following a predictable timeline (Bauer G. et al., 2021). Typically, the antibody response starts with the production of IgM by B lymphocytes, followed by the appearance of high-affinity IgG through a process called class switching. IgG levels usually peak around the 21st day after infection (Chung Y.S. et al., 2024) *(Figure 11)*.

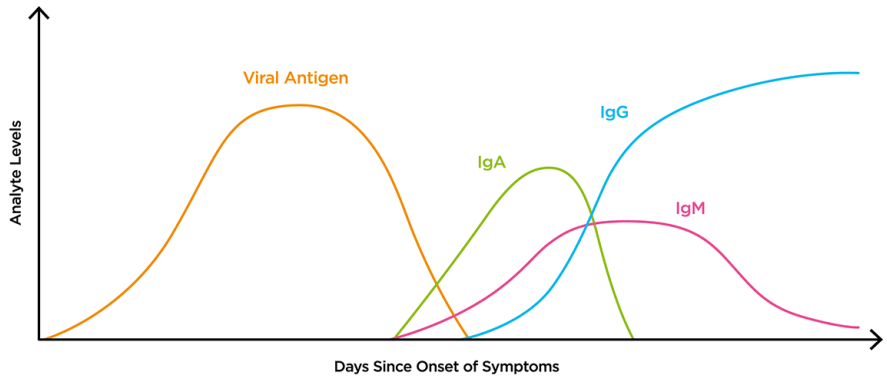

Figure 11 Classic Immune Response *(Tecan Trading AG, "Get Started with Ready-to-Use Coronavirus ELISA Kits")*

However, early serological studies on SARS-CoV-2 revealed that this pattern may not always be followed. In many cases, IgM is absent, delayed, or appears simultaneously with IgG (Liu L. et al., 2020). This atypical behaviour may result from individual differences in immune activation timing. When IgM production is delayed or IgG appears early, both classes can be detected in serum simultaneously, despite the initial activation of IgM-specific B cells (Bauer G. et al.,2021).

Additional complexities arise from the fact that IgM may persist beyond typical timelines, extending past the acute phase or reappearing upon reinfection, complicating the distinction between recent and past infections (Li Y. et al., 2025). Moreover, cross-reactivity between antibodies against seasonal human coronaviruses (such as HCoV-229E and HCoV-HKU1) and those against SARS-CoV-2 can cause false positives in serological tests, especially those targeting the S or N viral proteins (Chung Y.S. et al., 2024).

1.6 STRATEGIES FOR COVID-19 THERAPEUTIC AND VACCINATION MEASURES

From the very beginning of the pandemic, it became evident that the most vulnerable populations (i.e. elderly and individuals with pre-existing chronic conditions) required priority protection. The high incidence of severe disease and complications among these groups underscored the urgent need for targeted prevention strategies and early immunization (Abrignani MG et al., 2022).

During the early months of the global health emergency, when no specific therapies was approved for COVID-19, the medical community relied on repurposed drugs and convalescent plasma (Hurt AC. et al., 2021).

In March 2020, the U.S. Food and Drug Administration (FDA) launched the Coronavirus Treatment Acceleration Program (CTAP) to expedite the development and evaluation of promising therapies. This initiative mobilized internal divisions such as Centre for Drug Evaluation and Research (CDER) and Centre for Biologics Evaluation and Research (CBER), actively promoting clinical trials and dedicated research programs (FDA, 2020; FDA, 2023; FDA 2024). Simultaneously, the U.S. Department of Health and Human Services (HHS) recognized the public health emergency and authorized Emergency Use Authorizations (EUA) for drugs and biologics starting in April 2020 (HHS, 2020).

A structured approach was adopted in Europe as well: the European Medicines Agency (EMA) established the COVID-19 EMA Pandemic Task Force (COVID ETF), later restructured in 2022 as the Emergency Task Force Mechanism (ETFM) under Regulation (EU) 2022/123. Its dual mandate was to accelerate the regulatory process for novel

treatments and optimize the use of existing medicines for other indications (EMA, 2020; EMA, 2024; EMA, 2025).

By October 2021, CTAP had reviewed hundreds of clinical trials, resulting in emergency use authorization for 11 treatments and the full approval of the first COVID-19–specific drug. Simultaneously, the ETF supported the authorization of five therapeutic products in the European Union, including four neutralizing monoclonal antibodies or antibody combinations (FDA & EMA, 2021).

Additional research has explored the potential protective role of ACE2 blockers (i.e. enalapril, lisinopril, etc.) in COVID-19 disease progression (Sanaie S. et al., 2021).

1.6.1 Convalescent plasma and Monoclonal Antibodies

Convalescent plasma emerged as a leading early intervention during the SARS-CoV-2 pandemic. This passive immunization strategy involves transfusion of plasma from recovered individuals, containing neutralizing antibodies specifically directed at the virus. It was previously used successfully in other viral outbreaks, including H1N1 influenza, SARS, and Ebola (Casadevall A. & Pirofski L.A., 2020).

Plasma is collected via plasmapheresis, biologically processed to ensure safety, and rigorously tested for antibody titer (Hähnel V. et al., 2020) (*Figure 12*). It was deemed particularly promising when administered during the early phase of infection, while viral replication remains active. Based on these preliminary data, the FDA granted an EUA for convalescent plasma on August 23, 2020 (Joyner M.J. et al., 2021).

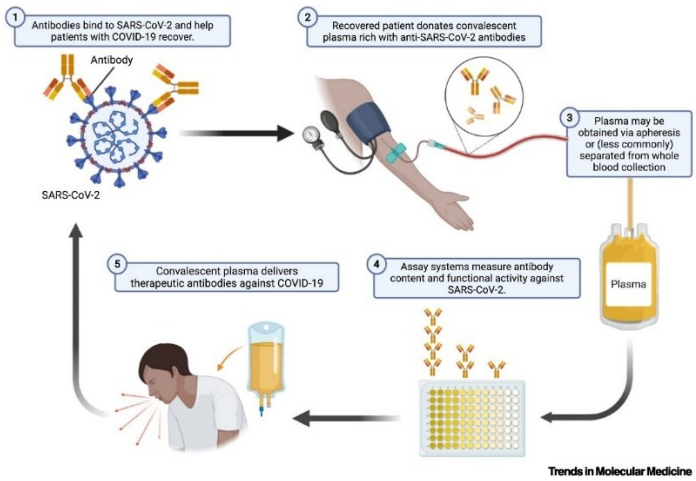

Figure 12 Steps of the convalescent plasma process: *collection from recovered patients, plasma separation, and administration to patients in treatment. (Senefeld JW et al., 2022)*

A large multicentre study involving over 35,000 participants found a significant mortality reduction when high-titer plasma was administered within three days of COVID-19 diagnosis (Joyner M.J. et al., 2021). Yet, systematic reviews and meta-analyses later concluded there was no significant clinical benefit in hospitalized patients with advanced disease (Klassen SA et al., 2021; Piechotta V. et al., 2023).
To overcome variability from individual donors, pooled hyperimmune immunoglobulin preparations were developed and purified industrially. These demonstrated more consistent antibody titers and favourable safety profiles (Rojas M. et al., 2021).

With the advent of monoclonal antibodies (mAbs) interest in convalescent plasma declined, mainly due to donor variability and its limited efficacy to early infection stages. These recombinant proteins specifically neutralize the virus and are produced from convalescent patient B cells or genetically humanized mice (Taylor PC et al. 2021). B-cell memory selection methods include flow cytometry, single-cell RT-PCR, and transgenic mouse models. Epitope-target mapping classifies mAbs into those targeting the receptor-binding motif, non-RBM RBD regions, and regions outside the RBD (Hwang YC. et al., 2022).
Their primary mechanism of action includes blocking Spike–ACE2 binding, enhancing phagocytosis, and activating the membrane attack complex (MAC) to induce apoptosis of infected cells (Suryadevara N. et al., 2021; Zhang T. et al., 2024).
Most effective mAbs target the Spike protein's RBD, though antibodies against the N-terminal domain (NTD) have also shown neutralizing capacity (Taylor PC et al., 2021; HwangYC et al., 2022).
Clinical trials have demonstrated that monoclonal antibody combinations—such as bamlanivimab–etesevimab and casirivimab–imdevimab—significantly reduce hospitalization and mortality in patients with mild-to-moderate COVID-19 (McCreary EK. Et al., 2022) (*Figure 13).*

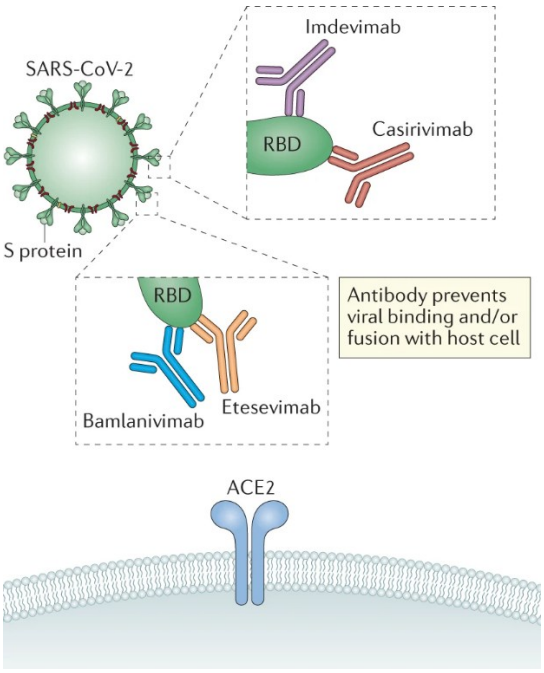

Figure 13 Neutralizing monoclonal antibodies targeting the spike RBD block ACE2 interaction preventing viral entry. *Casirivimab/imdevimab and bamlanivimab/etesevimab bind distinct RBD epitopes with high affinity, neutralizing SARS-CoV-2 through complementary mechanisms (Taylor PC et al., 2021)*

1.6.2 Vaccination Strategies Against COVID-19

The complex and heterogeneous immune response to SARS-CoV-2, along with the virus's ongoing evolution, has posed significant challenges in evaluating vaccine efficacy against emerging variants. This necessitates dynamic prevention strategies underpinned by molecular and serological monitoring. Understanding cross-reactivity and cross-protection mechanisms is essential for effective vaccine design and public health policy (Lancet Commission, 2021).

The pandemic swiftly overwhelmed healthcare systems, diverting attention from both acute and chronic disease management, and highlighting the critical importance of robust preventive immunization in restoring social and healthcare system resilience (Hacker KA.et al., 2021).

In May 2020, the U.S. government launched *Operation Warp Speed*, allocating approximately $18 billion to accelerate vaccine development. Five out of sixteen vaccine candidates reached Phase 3 trials by January 2021 (Hotez P. et al., 2021). The development and distribution of SARS-CoV-2 vaccines revolutionized the pandemic response, markedly decreasing hospitalization rates and severe complications (AbdelWareth et al., 2023). Despite naturally acquired immunity eliciting a protective

antibody response, vaccination remains strongly recommended—even for those with prior infection (Gallais F. et al., 2021; CDC, 2024).
At the time of writing, global primary vaccination coverage remains suboptimal: only 67% of the world population has completed the initial vaccine series, influenced by misinformation and vaccine hesitancy (AbdelWareth L. et al., 2023; WHO, 2024) while the global impact of COVID-19 remains severe: as of 2025, WHO reports over 7 million confirmed deaths (WHO, 2025)

Current SARS-CoV-2 vaccines are based on five primary platforms:
- live-attenuated or inactivated virus,
- nucleic-acid vaccines (RNA or DNA),
- viral vector vaccines (replicating and non-replicating),
- protein subunit vaccines,
- virus-like particle (VLP) vaccines.

Live-attenuated vaccines have demonstrated the capacity to induce trained immunity, an enhanced, nonspecific innate immune response with potential cross-protection—an attractive concept for future pandemic preparedness (Netea MG et al., 2023).

mRNA vaccines carry the genetic instructions that enable host cells to produce a viral antigen, thereby triggering a specific immune response. In the case of SARS-CoV-2, the main target is the Spike protein, which allows the virus to bind to the ACE2 receptor and enter human cells (Firouzabadi N. et al., 2023).
These vaccines are generally categorized into three main types:
- non-replicating mRNA,
- self-amplifying mRNA (saRNA),
- circular mRNA (circRNA).

In all cases, the messenger RNA is delivered into target cells via a carrier system — typically lipid nanoparticles (LNPs) — which protect the mRNA from degradation and facilitate its cellular uptake through endocytosis. (Reichmuth AM et al., 2016)
Cells that take up the LNPs- mRNA include both non-immune cells (such as muscle or skin cells at the injection site) and immune system cells like dendritic cells and macrophages located in nearby tissues or lymph nodes, reached via the lymphatic circulation. Once inside the cell, the mRNA is translated by ribosomes into viral proteins. Peptides derived from these proteins are displayed on the cell surface by major

histocompatibility complex class I (MHC-I) molecules, thereby activating $CD8^+$ cytotoxic T cell (Miyasaka M, 2022). At the same time, viral proteins released into the extracellular space can be phagocytosed by antigen-presenting cells (APCs) and presented via MHC class II molecules to $CD4^+$ helper T cells, which in turn activate B cells to produce specific antibodies (Fang E et al., 2022; Firouzabadi N. et al., 2023) (Figure 14).

The most widely used mRNA vaccines include Pfizer-BioNTech (BNT162b2), Moderna (mRNA-1273), and CureVac. The first one receiving authorization for commercial distribution was BNT162b2 (Fang E. et al., 2022). Among the most relevant, though rare, adverse effects cases of myocarditis and some forms of autoimmune vasculitis were reported (Sallard E. et al., 2023). Its original formulation was based on the genetic sequence of the wild-type virus, but it has been updated over time to address emerging variants with both bivalent and monovalent versions (Cao Y. et al., 2022; Fabiani M. et al., 2023). These updates were necessary to preserve vaccine efficacy, which was initially very high with two doses but showed a progressive decline over time and with the appearance of new variants — a phenomenon known as waning immunity (Firouzabadi N. et al., 2023).

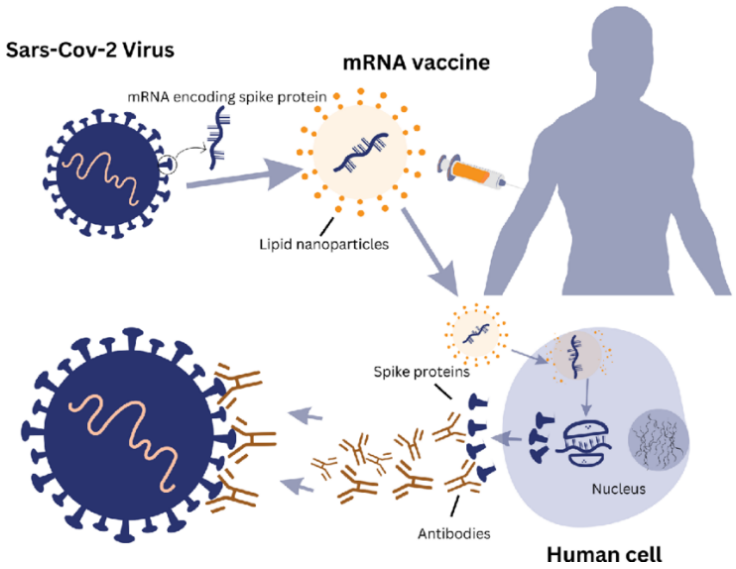

Figure 14 Schematic representation of the mechanism of action of mRNA vaccines against SARS-CoV-2*. Lipid nanoparticles (LNPs) deliver the mRNA into cells at the injection site, where it is translated into the Spike protein. The protein is presented via class I MHC molecules to cytotoxic T cells ($CD8^+$), and once released, it is captured by antigen-presenting cells (APCs) and displayed to helper T cells ($CD4^+$) through class II MHC molecules, thereby activating B cells to produce specific antibodies. (Aygün I et al., 2023)*

Adenovirus (Ads) Vaccines are non-replicating viral vector vaccines , valued for their rapid production, stability at standard temperatures, and ability to elicit both humoral and

cellular immunity. Notable examples include Vaxzevria (AstraZeneca, ChAdOx1 nCoV-19) and Jcovden (Primorac D. et al., 2022)

These vaccines are based on genetically modified, replication-deficient adenoviral vectors that deliver the gene encoding the Spike protein (Dong D. et al., 2025). The vectors are rendered replication-incompetent by deleting the *E1A* and *E3* genes, which are replaced by the Spike gene. They are produced in HEK293 cell lines that provide the missing genes in trans (Sakurai F. et al., 2022).

Upon intramuscular injection, the viral DNA enters host cells and is transported to the nucleus, where it is transcribed into mRNA and then translated into the Spike protein. The Spike protein is then presented via MHC-I to activate $CD8^+$ cytotoxic T cells or released into the extracellular space, captured by APCs, and presented via MHC-II to activate $CD4^+$ helper T cells, which subsequently stimulate B-cell antibody production.

Entry of the adenoviral vector is mediated through receptors such as CAR, CD46, DSG2, sialic acid, and heparan sulfate proteoglycans, depending on the serotype (Sakurai F. et al., 2022; Sallard et al., 2023). Both Jcovden and Vaxzevria have been withdrawn from use in Italy due to safety concerns (Sallard E et al., 2023).

Protein Subunit Vaccines: Nuvaxovid (NVX-CoV2373) vaccine (Novovax) is a recombinant protein vaccine against SARS-CoV-2. It uses a baculovirus-insect cell expression system to produce a purified prefusion-stabilized Spike protein, formulated with the saponin-based adjuvant Matrix-M™, which enhances both humoral and cellular immune responses (Heath PT et al., 2021).

This platform is a viable alternative to mRNA and viral vector vaccines, especially for individuals with contraindications to those technologies (Clothier HJ et al., 2024).

Inactivated Vaccines: represent a traditional and widely used platform against SARS-CoV-2, particularly in middle- and low-income countries, owing to low cost, scalability of production, and logistical stability (Verdecia M. et al., 2021).

CoronaVac comprises whole-virus particles that have been inactivated, incapable of replication but yet immunogenic. This broad antigenic exposure includes multiple viral proteins beyond the Spike protein, potentially conferring a wider immune response—although the magnitude of the antibody response is generally lower compared to mRNA vaccines (Verdecia M et al., 2021; Mat Yassim AS et al., 2025).

The original formulation was based on the Wuhan-Hu-1 strain and provided effective protection against severe disease and hospitalization. However, breakthrough infections occurred more frequently as variants emerged (Carabelli AM et al., 2023).

1.6.3 Emerging Strategies

- **Oral vaccines**, such as those developed by Vaxart and IosBio, utilize thermostable adenoviral vectors and can be administered in capsule form. Preclinical studies suggest they induce both systemic and mucosal immunity, including IgA production and T-cell responses
- **Intranasal vaccines**, currently in advanced development stages, use either adenoviral vectors or virus-like particles. For example, the Ad5-S.Mod intranasal aerosolized vaccine generated strong mucosal immunity in murine models, including IgA production and tissue-resident pulmonary T cells, providing complete protection against lethal SARS-CoV-2 challenge (Wang SY et al., 2023). These mucosal vaccines are designed to elicit tissue-resident memory immunity in the respiratory tract, which is crucial for early infection interception and reducing viral transmission. Additionally, inhalable neutralizing antibodies are under investigation to provide direct passive immunity in the airways for immediate viral neutralization

In the United States, the Project NextGen initiative allocated up to $5 billion to support vaccines capable of eliciting broad immunity against emerging SARS-CoV-2 variants and the wider family of epidemic-prone sarbecoviruses, vaccines that induce effective mucosal responses to prevent infection and transmission, and monoclonal antibodies resilient to viral evolution, providing a robust defense against future beta-coronavirus threats (Yang Z et al., 2022; Becerra X & Jha A, 2023)

1.6.4 COVID-19 Vaccination Strategies and Priorities in Italy

The national COVID-19 vaccination campaign in Italy was officially launched on December 27th, 2020, followed by the formal adoption of the National Strategic Plan for COVID-19 Vaccination on March 12th, 2021. This plan implemented a risk-based prioritization framework, targeting population groups based on individual vulnerability and public health impact. The rollout proceeded as follows:

1. Healthcare and social care professionals;
2. Residents and staff of long-term care facilities (LTCFs);

3. High-risk individuals, including the elderly, immunocompromised patients, and those with chronic co-morbidities;
4. School personnel and law enforcement officers;
5. The general population, in descending age order.

Authorized and distributed vaccines included mRNA-based platforms (Comirnaty by Pfizer-BioNTech and Spikevax by Moderna), non-replicating viral vector vaccines (Vaxzevria by AstraZeneca and Janssen by Johnson & Johnson), and a pediatric formulation of Comirnaty. By January 2022, over 80% of the Italian population had received at least one vaccine dose (Istituto Superiore di Sanità, 2022).

1.7 DIAGNOSIS

The most recent pandemic, caused by SARS-CoV-2, had an unprecedented global impact, emphasizing the urgent need not only for preventive and therapeutic interventions but also for accurate diagnostic methods capable of rapidly performing differential diagnosis from other pathogens, crucial for monitoring infection dynamics (Illi B. et al., 2020; Hurt AC et al., 2021; Rolf M., 2023). Early in the pandemic, the lack of distinguishing clinical features for SARS-CoV-2 posed a significant challenge to the scientific community, necessitating the rapid deployment of reliable diagnostic strategies. Initially, diagnoses were based on clinical presentation and exclusion of other pathogens causing similar symptoms, until next-generation sequencing (NGS) enabled SARS-CoV-2 identification and typing (Huang C et al., 2020).

The main diagnostic techniques used for the detection of SARS-CoV-2 are shown in *Figure 15*.

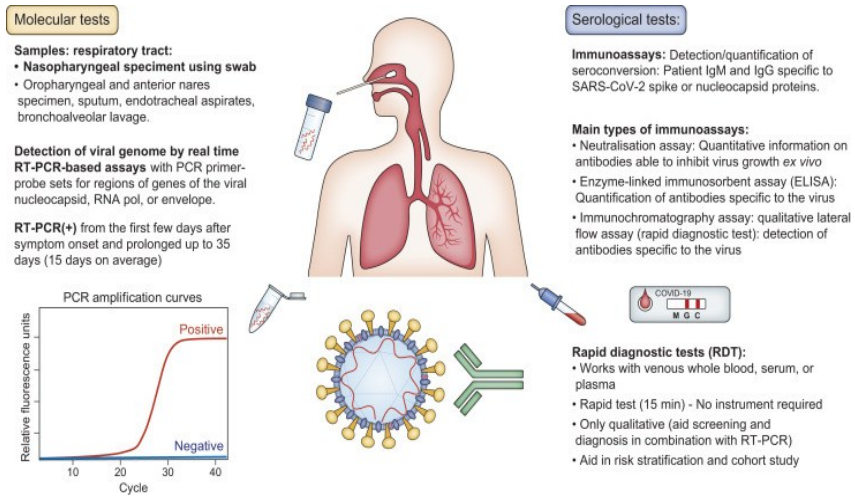

Figure 15 The main diagnostic techniques used for the detection of SARS-CoV-2 *(Asselah T et al., 2021)*

Throughout the pandemic, molecular testing based on the detection of viral RNA via RT-PCR (Reverse Transcript-Polymerase Chain Reaction) using nasopharyngeal swabs were considered the gold standard. They had a central role in the diagnosing of active infections but, most importantly, for the identification of asymptomatic carriers (Illi B. et al., 2020; Rolf M., 2023). According to Liu L. et al. (2020), RT-PCR testing is ideally performed within 11 days of symptom onset. Specimen collection can be performed from the upper respiratory tract (nasopharyngeal swab) or the lower respiratory tract (sputum), the latter demonstrating greater sensitivity. The diagnostic process involves reverse transcription

followed by amplification using specific primers and probes targeting highly conserved genomic regions of SARS-CoV-2(Chung YS et al., 2024). Even though the high sensitivity of molecular tests limitations of this these techniques are also present. As an example, a negative result does not necessarily rule out infection, particularly if the sample is collected at a suboptimal stage of disease progression. In addition, they are not able to provide information on past infections as well as requiring considerable logistical and organizational efforts (Kucirka LM et al., 2020; Larremore DB. et al., 2021). Many countries also adopted antigen swabs and saliva-based tests, which, despite lower sensitivity, offered a more practical approach for frequent screening. These rapid testing strategies enabled prompt isolation of positive cases and effective contact tracing, supporting targeted outbreak containment

1.8 SEROLOGICAL ASSAYS

Serological assays can detect specific IgG and/or IgM antibodies, confirming past infections even in asymptomatic individuals, as well as monitor the duration and effectiveness of both natural and vaccine-induced immune responses over time (Bauer G. et al., 2021). For many years, ELISA served as the benchmark for serodiagnosis. However, newer, faster, and more cost-effective multiplex technologies are increasingly complementing ELISA. A key advantage of multiplex assays is their ability to simultaneously analyse multiple analytes from minimal sample volumes. In contrast, traditional ELISA requires separate assays for each target antigen.

The integration of reliable diagnostic methodologies proved to be essential—not only to identify active infections but also for evaluating immune responses and vaccine effectiveness (Peeling RW. et al., 2022).

1.8.1 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a widely used immunoenzymatic technique for the detection and quantification of antigens or antibodies in biological samples. The assay relies on the specific interaction between an antigen and its corresponding antibody. Either the antigen or antibody is immobilized on a solid surface (Hornbeck PV. 2015), followed by the addition of an enzyme-conjugated detection molecule. The enzyme catalyzes a substrate reaction that produces a detectable signal, which is proportional to the analyte concentration except for

competitive ELISA whose intensity is inversely proportional to the concentration of the target analyte (Aydin S et al., 2025). The colour change is accurately detected by a spectrophotometer, which provides as output an absorbance value expressed in optical density (OD). The antibody titer is calculated by interpolating the OD obtained respect to cut-off value (semi-quantitative ELISA), as a concentration/unit (quantitative ELISA) or as presence or not of a signal (qualitative ELISA).

Different ELISA formats shown in figure 16 are used depending on analytical requirements.

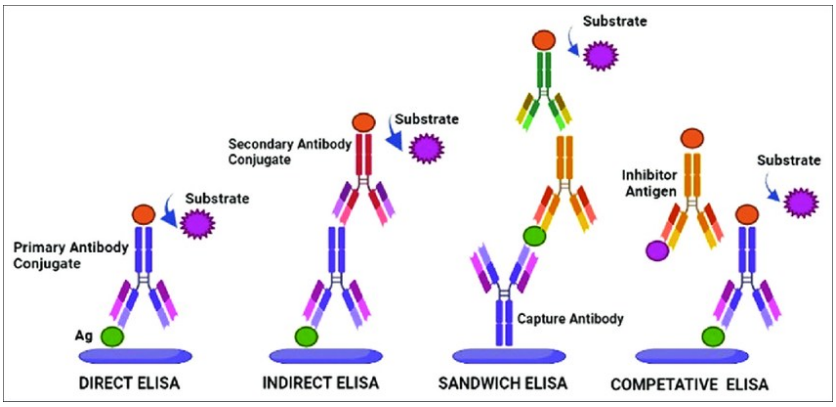

Figure 16 Schematic representation of the main ELISA formats: direct, indirect, sandwich, and competitive. *Each configuration differs in the way the antigen or antibody is immobilized and detected, influencing assay sensitivity and specificity. 1. Direct ELISA: Enzyme-conjugated antibody binds directly to immobilized antigen; simple but less sensitive. 2.Indirect ELISA: Unlabelled primary antibody binds the antigen, followed by enzyme-conjugated secondary antibody; improves sensitivity via signal amplification; 3. Sandwich ELISA: Two antibodies recognize different epitopes of the same antigen; ideal for low-abundance targets with high specificity. 4. Competitive ELISA: Sample antigen competes with labelled antigen for limited antibody binding. Here, the signal is inversely proportional to antigen concentration. This technique is useful for small molecules or haptens* (*Khan M* et al., 2022)

1.8.2 LUMINEX TECHNOLOGY

The Luminex xMAP® technology is a high-throughput multiplex platform that enables the simultaneous detection of up to 500 analytes within a single sample. The assay uses internally dyed polystyrene microspheres (5.6–6.5 µm), each labeled with a unique ratio of two fluorescent dyes for bead identification, while a third fluorescent signal measures analyte binding. (Dunbar S.A, 2006). Each antigen of interest is covalently coupled to a specific bead population, creating a discrete multiplex array of analytes within a single reaction well (Cameron A et al., 2021). The Luminex reading is achieved through a dual-laser flow cytometry-based detection system: a red laser (635 nm) identifies the bead set based on internal dye signature, while a green laser (532 nm) quantifies the PE signal, which is proportional to the amount of bound analyte (Luminex Corporation, *xMAP® Cookbook*, 5th ed. (2023)).

The key distinction between Luminex and ELISA lies in their capture systems. In ELISA, the target molecules are immobilized on the surface of multi-well plates, whereas Luminex captures analytes on suspended microspheres. Luminex employs fluorescence as a readout, while ELISA relies on enzyme-mediated colorimetric reactions that produce a measurable signal upon substrate conversion (Mountjoy KG., 2021) *(Figure 17)*

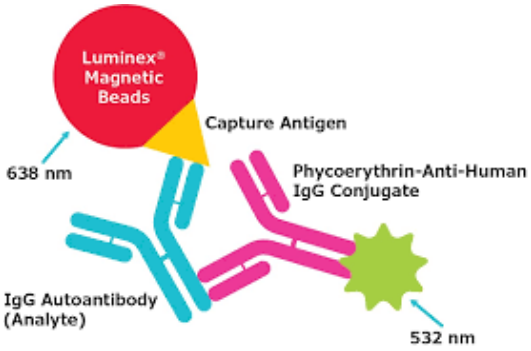

Figure 17 Schematic representation of the Luminex MILLIPLEX® SARS-CoV-2 Antigen Panel principle*. Fluorescent microspheres, each coated with a specific SARS-CoV-2 antigen, capture antibodies present in the sample; the antigen–antibody complex is then detected using a fluorophore-conjugated secondary antibody* (*Sigma-Aldrich, "Multiplex Analysis of Autoantibodies"*).

1.8.3 MESO SCALE DISCOVERY (MSD) Platform

The MSD platform is an advanced immunoassay technology that utilizes electrochemiluminescence (ECL) for the sensitive and simultaneous quantification of multiple analytes within a single biological sample. The assay is performed on designed multi-array plates containing carbon electrodes, each spot pre-coated with capture antibodies specific for the analytes of interest (Han Z et al., 2021) *(Figure 18).*

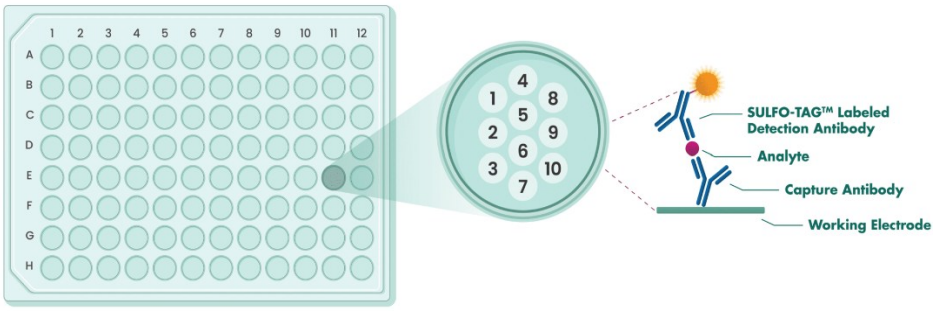

Figure 18 MSD plate *(Reaction Biology, "Multiplex Immunoassay Service")*

A detection antibody, labelled with the SULFO-TAG® ECL label, is then introduced, forming a sandwich complex. When an electrical potential is applied to the plate, the

SULFO-TAG® emits light in a redox reaction, producing a luminescent signal that is measured by a dedicated MSD instrument (Marchese RD et al., 2009).

This signal is directly proportional to the amount of analyte present in the sample, enabling highly sensitive and quantitative detection. The technology supports both single plex and multiplex formats, allowing simultaneous measurement of multiple targets from minimal sample volumes with high dynamic range and reproducibility.

2 AIM OF THE STUDY

The SARS-CoV-2 pandemic caused an unprecedented global health crisis starting in late 2019 and lasting through the early 2020s. This highlighted the critical need for reliable, sensitive, and reproducible diagnostic and serological tools capable of supporting both patient management and the assessment of immune responses induced by natural infection or vaccination. The measurement of specific antibodies targeting viral antigens, such as the Spike protein and the Nucleocapsid protein, is essential for understanding individual and population immunity, as well as for monitoring vaccine efficacy and tracking the evolution of viral variants.

In recent years, alongside traditional serological assays such as ELISA, technologically advanced platforms based on multiplexing principles have emerged, enabling the simultaneous measurement of multiple analytes within a single sample. Among these, Luminex and MSD systems have demonstrated significant potential in terms of sensitivity, dynamic range, and reduction of sample and reagent volumes, representing particularly promising tools for large-scale sero- profiling studies.

The primary aim of this study is to comparatively evaluate the analytical performance of three serological platforms — ELISA, Luminex, and MSD — for the quantification of SARS-CoV-2-specific antibodies. The objective is to identify limitations and complementarities of each method.

Specifically, the use of multiplex methodologies seeks to verify their applicability as reference tools for serodiagnosis and immune surveillance, not only in the context of COVID-19 but also as a model for managing future infectious disease emergencies. The comparative approach adopted also aims to contribute to the standardization of bioanalytical methods, promoting better data interoperability between laboratories and more accurate interpretation of serological results.

This work intends to demonstrate the added value of multiplex systems compared to conventional assays, highlighting how their integration can enhance the efficiency, reliability, and informational capacity of immunological analyses. Such a comparative study represents a crucial step towards the validation of innovative methodologies for broader applications in research, clinical practice, and public health.

3 MATERIALS AND METHODS

3.1 Samples: Commercial human serum panels

50 human sera from commercial panels have been used in this study (Panels D and F from Access Biological, LLC, Vista, CA) (Table 1). Panel D consists of sera collected from 34 SARS-CoV-2 PCR-positive donors during the first wave of infections in the United States between March 14th and April 25th, 2020 while the panel F includes 16 donors, all ages ranged from 25 to 66 years old, all of whom exhibited symptomatic COVID- 19. Serum from all samples was screened negative for syphilis and antibodies to HIV-1/2, HCV and nonreactive for hepatitis B surface antigen (HBsAg). Each sample was analyzed in parallel using the three methods for the detection of specific antibodies directed against three viral antigens: the N protein, the RBD of the S protein, and the full-length Spike protein.

Sample ID – Panel D	
SB137447	SB137551
SB137448	SB137567
SB137455	SB137574
SB137460	SB137576
SB137469	SB137578
SB137493	SB137579
SB137495	SB137582
SB137500	SB137583
SB137501	SB137589
SB137503	SB137593
SB137508	SB137594
SB137515	SB137597
SB137517	SB137605
SB137525	SB137631
SB137528	SB137694
SB137545	SB137695
SB137546	SB137701

Sample ID – Panel F
5530341113
5530341114
5530341115
5530341128
5530341249
5530341283
5530341293
5530341348
5530341355
5530341401
5530341566
5530341596
5530341625
5530341647
5530341683
5530341684

Table 1. List of the 50 human serum samples (n = 50) used in this study, sourced from commercial panels D (left table) and F (right table) *provided by Access Biological, LLC (Vista, CA, USA)*

3.2 ELISA

3.2.1 Materials

Recombinant antigens N protein and RBD were purchased from SinoBiological while protein S1 was ordered from eEnzyme LLC. Coating Buffer: prepared by dissolving 1 capsule of Carbonate- Bicarbonate (pH 9.3 - 9.9) (Sigma Aldrich) in 100 ml of distilled water 0.05M. Wash Buffer: prepared by diluting Tris Buffered saline (TBS) –0.05%Tween 20 (20 X) in Milli-Q water to reach a 1X concentration. Blocking & Dilution Buffer: prepared by dissolving non-fat dry milk (NFDM) (EuroClone) in Wash Buffer to reach the final concentration of 5%. HRP-conjugated Goat anti-human IgG secondary antibody (Bethyl laboratories). 0.5 M HCl, Hydrochloric acid (Fisher Scientific). 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma Aldrich). Positive Control: anti-spike RBD (SARS-CoV-2/COVID-19) human monoclonal antibody (eEnzyme LLC), anti-COVID-19 & SARS-CoV S glycoprotein and anti-COVID-19 & SARS-CoV Nucleoprotein (Absolute Antibody). Negative Control: Human Serum Minus IgG/IgM/IgA depleted (Merk Life Science).

3.2.2 Method

In this work an in-house optimized indirect ELISA was used. Firstly, a clear 96 well plate is coated with 1 μg/ml of the recombinant antigen of interest by diluting it in Coating Buffer obtaining the Coating solution. The plate is incubated overnight at +4°C. The next day the coating solution is washed out by using wash buffer solution and a blocking solution is added to each well to prevent non-specific antibody binding in the next steps. While the plates were incubated from 1 to 3 hours at 37°C, samples are prepared to be 2-fold serially diluted, ranging from 1:100 to 1:51200. Dilutions are performed in a deep well plate during blocking incubation time; each sample is tested in duplicate. Positive and negative controls are prepared using the dilution buffer, with the positive control adjusted to a final dilution of 1:8000 for RBD detection and 1:1000 for S1 and N. Once the blocking time is completed, plates are washed as described above and diluted samples/controls are transferred to the coated plate. An incubation of 1 hour at 37°C is now required to permit the binding of specific antibodies to the investigated antigen.

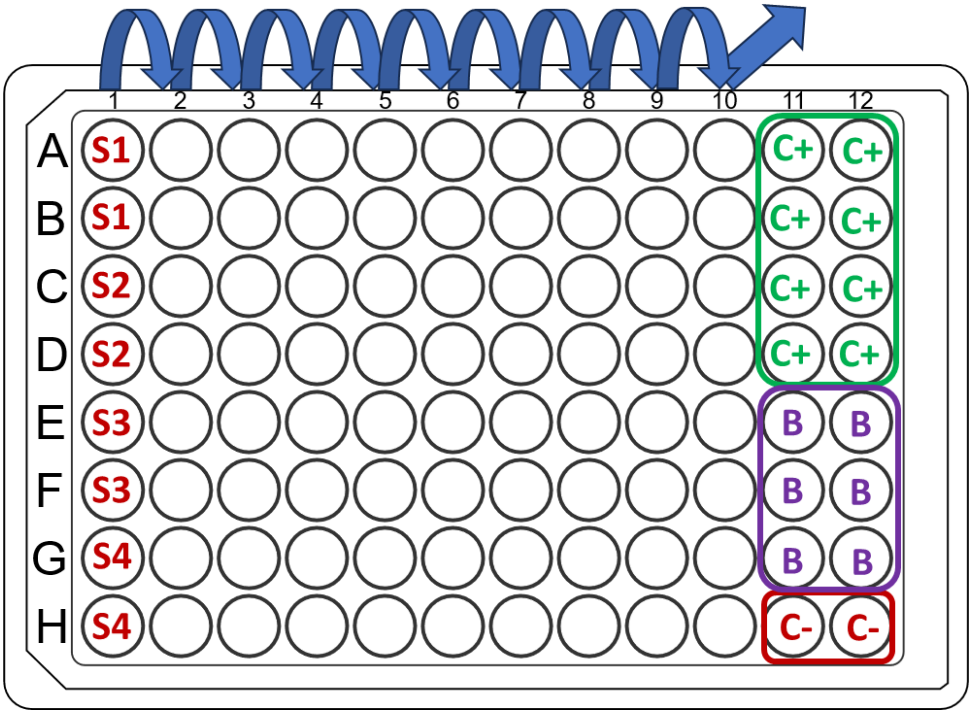

Figure 19 Schematic representation of an ELISA plate. *Samples (S1, S2, S3, S4), diluted 2-fold from column 1 to 10, on the plate coated with the antigen of interest. Positive control (C+) is shown in green, while negative control (C-) is shown in red. Illustrations by the author.*

When the incubation is completed, plate is washed and detection solution added, containing an HRP-conjugated secondary anti-human IgG antibody with a final dilution of 1:100,000. The reaction is incubated for 30 minutes at 37°C. Afterwards, a final wash step is performed and TMB solution is added. TMB is a chromogenic substrate that reacts with the peroxidase of the antibody producing a blue coloration whose intensity increases proportionally to the amount of antibodies present in the analysed serum. The reaction must occur in the dark and after 20 minutes the reaction is stopped using 0.05 M HCl. Plate's signal is acquired using a 96-well spectrophotometer with a wavelength of 450 nm.

3.2.3 Data analysis

The ELISA titre is expressed as the highest dilution able to provide an absorbance value greater than the cut-off value. The latter is expressed as an OD value obtained by using the 6 blank replicates. The ELISA duplicates, must have a Coefficient of Variation (CV) $\leq 20\%$.

3.3 LUMINEX XMAP® ASSAY

3.3.1 Materials

All reagents were provided within the MILLIPLEX® SARS-CoV-2 Antigen Panel (IgG 3-Plex customized; Luminex xMAP® Technology), including antigen-coated magnetic beads (S1, RBD, and N), positive and negative control sera, assay buffer, wash buffer, and PE-conjugated anti-human IgG detection antibody

3.3.2 Method

The Luminex assay was performed according to standard procedures for bead-based multiplex immunoassays. Briefly, the antigen magnetic beads coated with S1, RBD and N antigen were vortexed and sonicated before being pooled and adjusted to the appropriate volume. Wells were pre-washed with wash buffer to remove any residues and prepare them for the assay.

1:300 diluted serum samples were added to the wells in duplicate along with assay buffer, followed by the addition of the bead mixture. The plates were sealed and incubated for 2 hours under shaking conditions (260 rpm) at room temperature to allow antibodies in the samples to bind to the beads. After incubation, the wells were washed several times using a magnetic washer to remove unbound material.

A PE-conjugated secondary antibody was then added and incubated for 90 minutes to detect bound IgG. Following a final wash, Sheath Fluid was added, and the plates were shaken briefly before reading on the Luminex instrument.

3.3.3 Data Analysis

The plates were read on the Luminex instrument- performed by setting the instrument parameters to a bead count ≥50, selecting the corresponding bead regions used in the assay, and applying a horizontal reading direction from well A1 to A12- which measures the fluorescence emitted by the PE-conjugated detection antibody bound to the beads. Data are expressed as Median Fluorescence Intensity (MFI) for each bead region, which reflects the concentration of the target antibody in the sample. Fluorescence intensity, corresponding to IgG concentration, was measured for each sample. Assay validity was confirmed by ensuring adequate bead counts (at least 50 per region)

3.4 Meso Scale Discovery (MSD) Assay

3.4.1 Material

All reagents and control were provided by kit MSD V-PLEX SARS-CoV-2 Panel 1 (MSD, USA) and includes Sulfo-TAG Anti-Human IgG Antibody, Diluent 100, MSD Wash buffer 20 X subsequently diluted at working concentration of 1X, Blocker A, MSD Phosphate buffer 5 X, MSD GOLD Read Buffer B. Serology control Pack and Reference Standard to generate a 7-point calibration curve (prepared through 4-fold serial dilutions and a blank) (Figure 20).

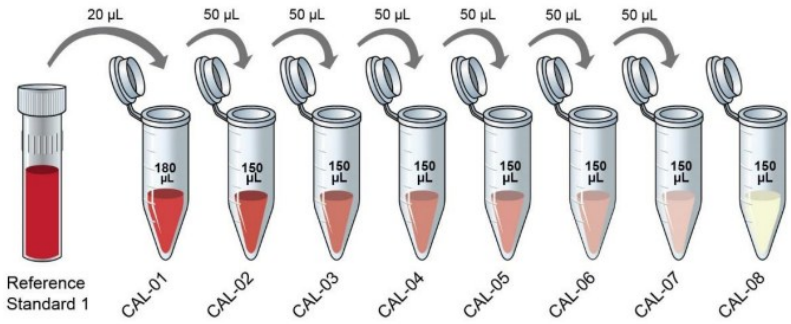

Figure 20 Construction of MSD 7- point calibration standard curve *(Meso Scale Diagnostics, "V-PLEX COVID-19 Serology Assays Insert")*

3.4.2 Method

The assay was performed following the manufacturer's instructions for the MSD platform. Briefly, the wells were first blocked with the appropriate blocking solution and incubated under shaking (260 rpm) conditions at room temperature to prevent non-specific binding. After removal of the blocking buffer, diluted serum samples and calibrators were added at final concentration of 1:500 in Diluent 100 to the designated wells and incubated to allow antibody binding.

Following this incubation, the plates were washed several times with Wash Buffer to remove unbound material. The SULFO-TAG-conjugated anti-human IgG detection antibody was then added at the concentration 1:200, and the plates were incubated again under the same conditions. After a second series of washing steps, the Read Buffer was added immediately prior to electrochemiluminescence reading using the MSD QuickPlex SQ 120 instrument.

3.4.3 Data Analysis

The electro chemiluminescent signal was measured in Relative Light Units (RLU) and analyzed with the MSD Discovery Workbench software. Sample concentrations were interpolated from the standard calibration curve and expressed as Arbitrary Units per millilitre (AU/mL) or, when applicable, as Binding Antibody Units per millilitre (BAU/mL). The assay was considered valid when internal controls were within the acceptable range and the CV between replicates did not exceed 20%.

4 RESULTS

To assess the degree of agreement among the three analytical platforms employed for SARS-CoV-2 antibody detection (ELISA, MSD, and Luminex), a Spearman correlation analysis was performed on 50 human serum samples derived from commercial panel. Correlation matrices shown in figure 21 are created based on the analyzed recombinant antigen: N, S1 and RBD.

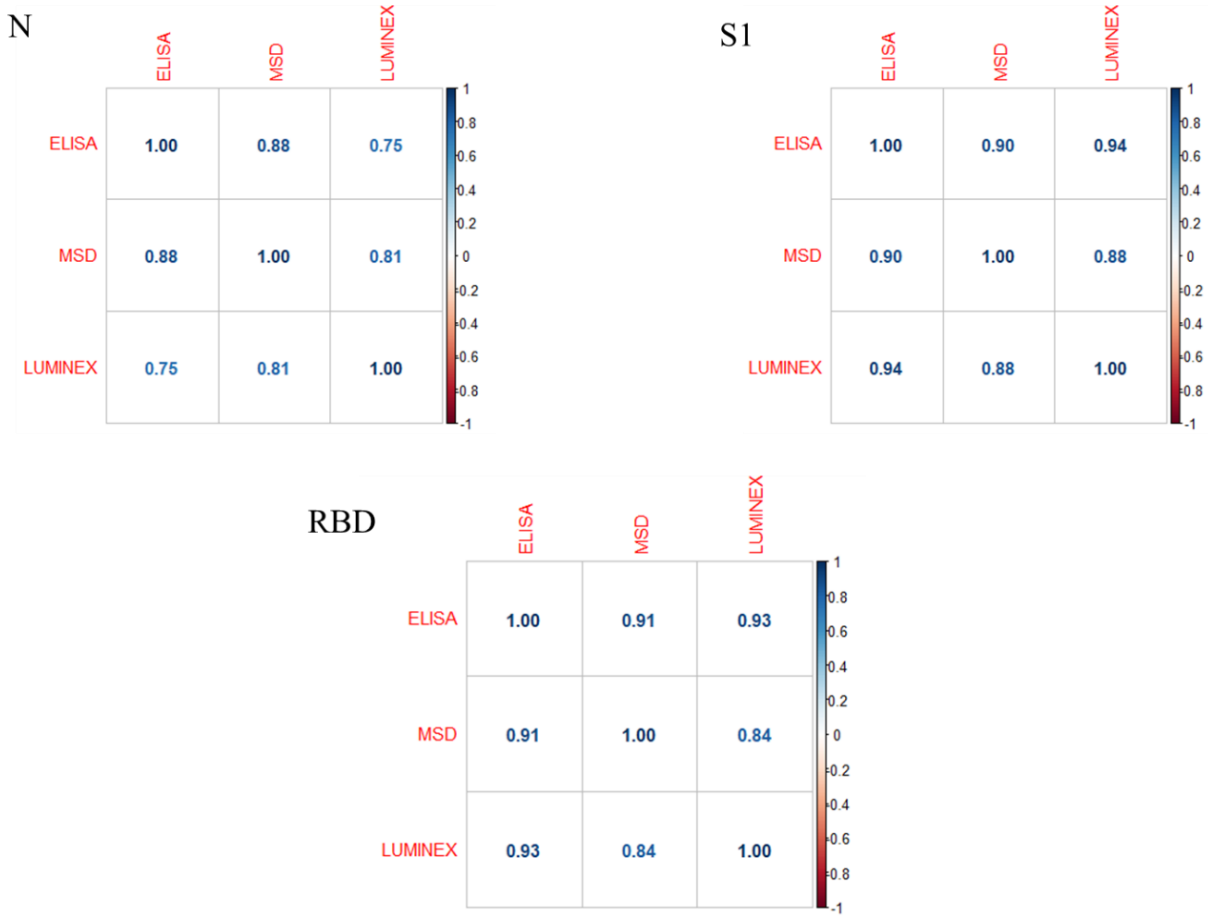

Figure 21 Correlation Matrix between COVID 19 main antigens. *Upper matrices report the N and S1 correlation values from left to right, respectively, while the lower represents the results obtained for RBD analysis. The correlation is quantified by displaying the Spearman correlation coefficient (r). The r has been calculated between the results obtained using the three analytical methods (ELISA, MSD, and Luminex), indicated on the horizontal and vertical axes. The diagonal values represent the perfect correlation (r = 1) of each assay with itself, while the off-diagonal values indicate the degree of correlation among the different platforms. The color scale on the right illustrates the strength and direction of the correlation, ranging from –1 (negative correlation) to +1 (positive correlation). Across all analyzed groups, a strong concordance was observed among the methods, with particularly high coefficients between ELISA and Luminex.* ***Legend:*** *n = 50 samples; Spearman correlation coefficients (r). Interpretation: r < 0.4 = low, 0.4–0.59 = moderate, 0.6–0.79 = moderately high, ≥ 0.8 = high. Numbers are color-coded according to the strength of the correlation.*

The overall degree of agreement among the three methods when tested against the N protein was high, with the strongest correlation observed between ELISA and MSD (r = 0.88). A slightly lower, though still robust, correlation was observed between ELISA and Luminex (r = 0.75). These findings indicate a good overall comparability between

platforms in detecting antibodies against the N antigen, with MSD displaying the highest inter-assay consistency.

As illustrated for the anti-S1 antibody correlations, all three assays exhibited stronger mutual agreement, with r values ranging from 0.88 to 0.94. The correlation between ELISA and Luminex was particularly high (r = 0.94), followed by ELISA–MSD (r = 0.90) and MSD–Luminex (r = 0.88). This finding suggests that the detection of antibodies directed against the S1 region of the spike protein is highly reproducible across different assay formats.

For the anti-RBD protein, a consistently strong linear relationship was found among all methods, with Spearman's r values ranging between 0.84 and 0.93. The correlation was particularly robust between ELISA and Luminex (r = 0.93), followed by ELISA and MSD (r = 0.91), while a slightly lower correlation was observed between MSD and Luminex (r = 0.84). These results indicate a high degree of analytical comparability and confirm that all three methods are capable of quantifying anti-RBD antibody levels.

To graphically visualize the previously described results, correlation plots were generated for each antigen and assay combination (Figures 22- 24).

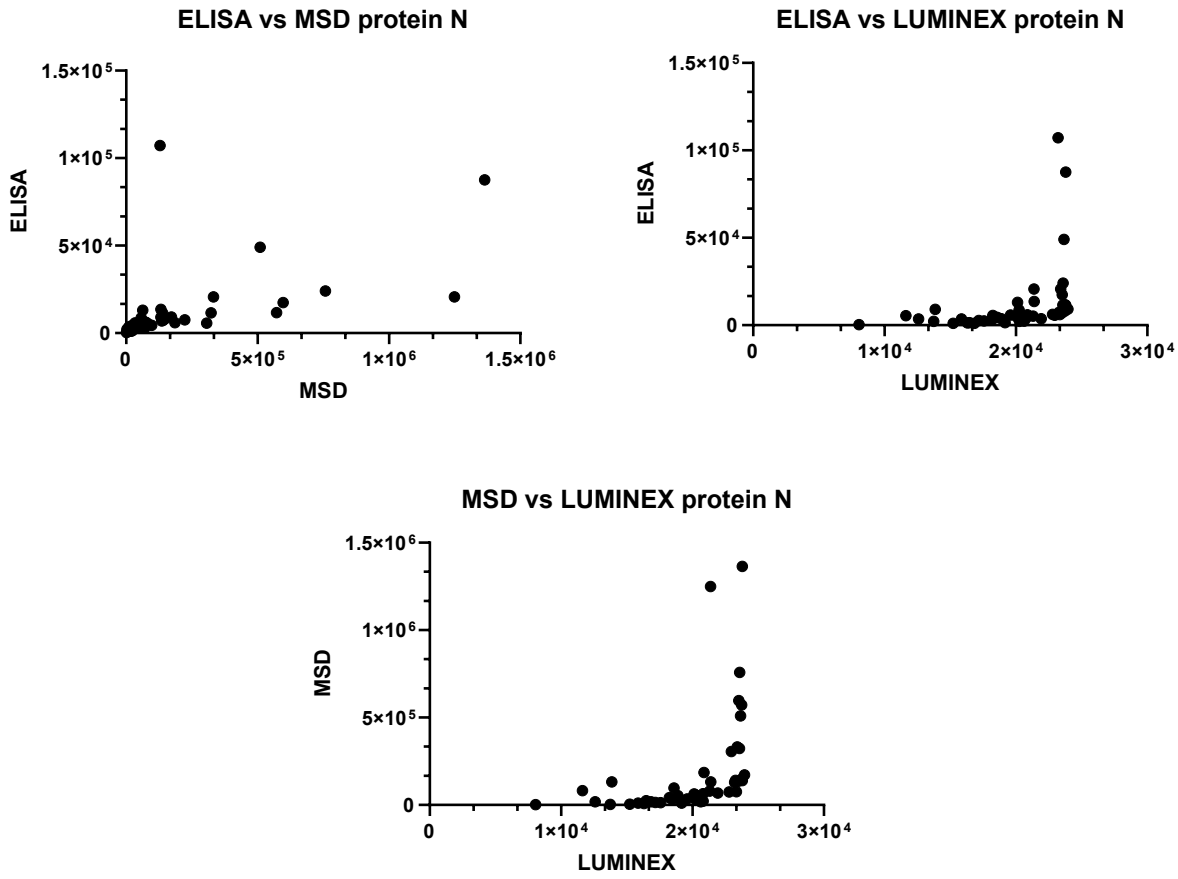

Figure 22 Correlation plot for the SARS- CoV-2 N protein. *Correlation plot comparing serological measurements for the N protein obtained with ELISA, MSD, and Luminex on 50 serum samples. Each panel compares two platforms: ELISA vs MSD (top left), ELISA vs Luminex (top right), and MSD vs Luminex (bottom). The quantitative values obtained with the three assays (ELISA, MSD and Luminex) were plotted on the x- and y-axes, allowing direct pairwise comparison between methods. Each dot represents a single serum sample, positioned according to the antibody levels measured by the two corresponding assays. Most samples show values near zero, while a few high-response outliers are roughly monotonic across platforms. The spread of points increases at higher values, indicating that differences between methods become more apparent in samples with strong responses.*

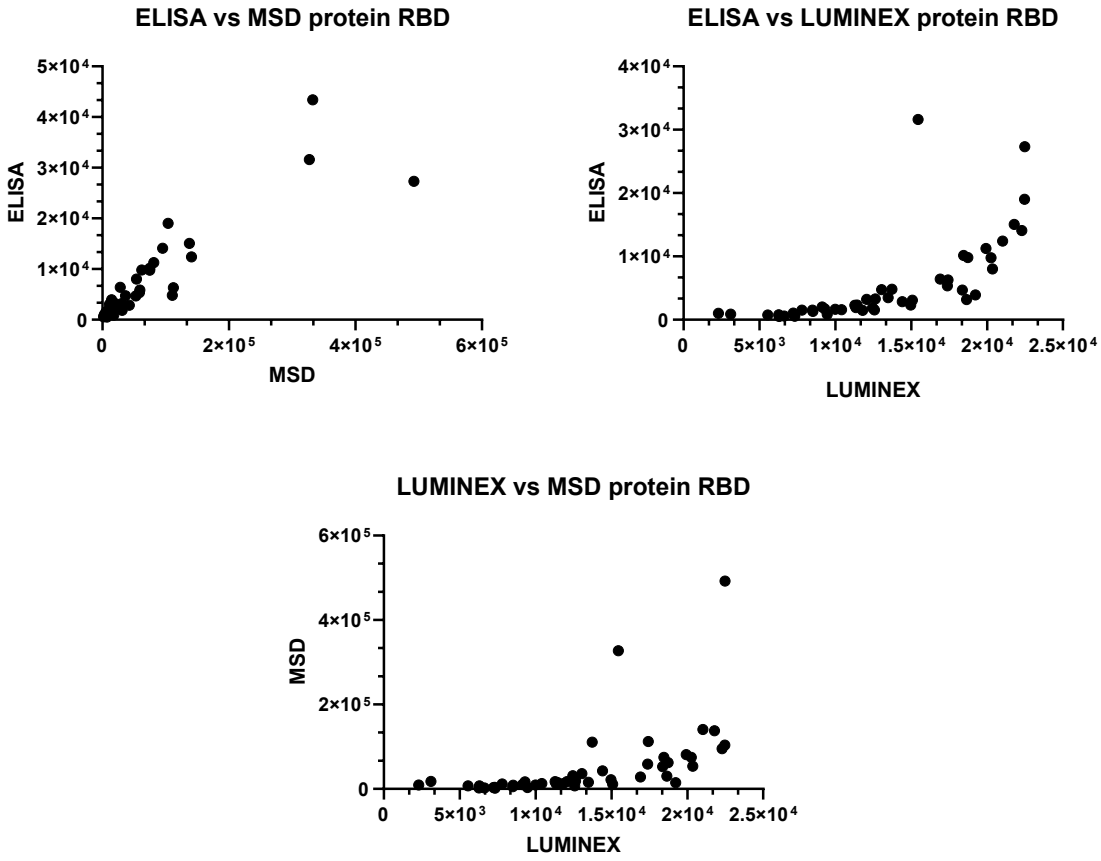

Figure 23 Correlation plot for the SARS- CoV-2 RBD protein. *Correlation plot comparing serological measurements for the RBD protein obtained with ELISA, MSD, and Luminex on 50 serum samples. Each panel compares two platforms: ELISA vs MSD (top left), ELISA vs Luminex (top right), and MSD vs Luminex (bottom). Each dot represents a single sample, allowing assessment of the distribution of the data and the correlation between variables. The plots also helped to identify possible outliers and visualize overall monotonic trends within the dataset. Most samples show low signals, while a few high-response outliers display roughly monotonic values across platforms. The spread of points increases with signal intensity, highlighting differences in the dynamic ranges and sensitivity of the methods*

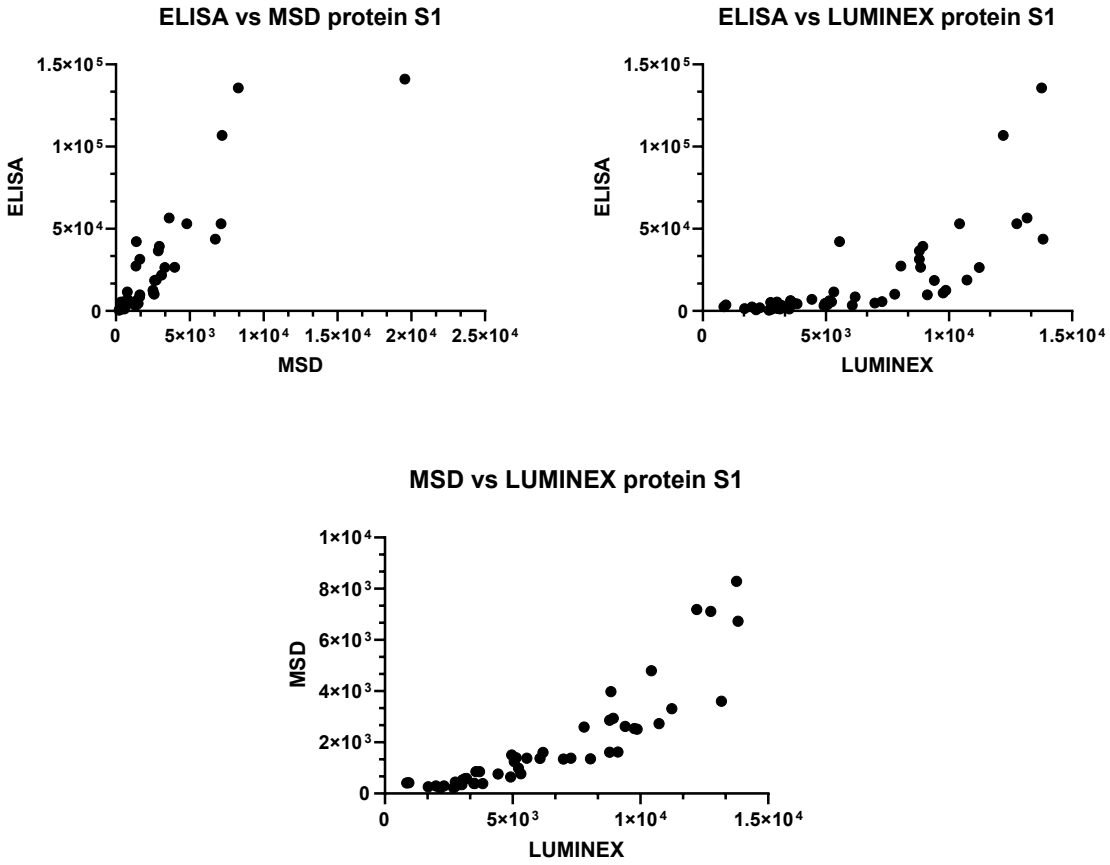

Figure 24 Correlation plot for the SARS- CoV- 2 S1 protein *Correlation plot comparing Serological measurements for the S1 protein obtained with ELISA, MSD, and Luminex on 50 serum samples. Each panel compares two platforms: ELISA vs MSD (top left), ELISA vs Luminex (top right), and MSD vs Luminex (bottom). Each dot represents a single sample, allowing assessment of the distribution of the data and the correlation between variables. The plots also helped to identify possible outliers and visualize overall monotonic trends within the dataset. Most samples show values near zero, indicating weak or absent responses, while a few high-response outliers exhibit roughly monotonic behavior across platforms. The ELISA–MSD comparison shows greater dispersion, suggesting weaker agreement, whereas comparisons involving Luminex display points more aligned along the diagonal. Point spread increases at higher values, highlighting differences between methods in samples with stronger S1 responses.*

5 DISCUSSION AND CONCLUSION

The SARS-CoV-2 pandemic, declared global health emergency by the WHO in March 2020, posed an unprecedented challenge to the international scientific community. The urgent need to rapidly understand viral biology, immune responses, and the duration of natural and vaccine-induced protection emphasized the importance of developing sensitive, rapid, and reliable diagnostic methods. While RT-PCR initially served as the reference for direct viral detection, the widespread implementation of vaccination campaigns highlighted the growing demand for serological tools capable of monitoring antibody responses over time and evaluating their functional quality. Traditional immunoassays, such as ELISA, have long provided a foundation for quantifying SARS-CoV-2-specific antibodies. However, this assay is limited in their ability to assess multiple antigens simultaneously and larger sample volumes is required. Multiplex immunoassays, including Luminex and MSD, have emerged as powerful alternatives, allowing simultaneous measurement of multiple immunological parameters in a single run. The Luminex platform, a bead-based fluorescent multiplex assay, and the MSD platform, based on electrochemiluminescence, have demonstrated high accuracy, sensitivity, and reproducibility (Kenny et al., 2022; Roy et al., 2023). Roy et al. (2023) developed a Luminex-based multiplex assay capable of detecting anti-Spike, anti-N, and anti-RBD IgG antibodies with >98% sensitivity and specificity, while Shengule S. et al. (2024) validated a nine-plex MSD assay for simultaneous detection of antibodies against multiple viral antigens and variants (Shengule S et al., 2024). Comparative studies indicate that, despite technical differences — with Luminex offering flexible microsphere selection and MSD providing superior analytical sensitivity — both platforms deliver comparable performance for serological monitoring and immune profiling (Cox et al., 2023).

In the present study, fifty human serum samples positive for SARS-CoV-2 were analyzed using ELISA, MSD, and Luminex to quantify anti-S, anti-RBD, and anti-N antibodies. Spearman correlation analysis revealed high concordance across methods, particularly for S and RBD antigens, with correlation coefficients frequently exceeding 0.9.

The improved correlation for RBD relative to N is likely attributable to the higher immunogenicity and structural stability of the RBD antigen, which may result in a more consistent antibody recognition pattern among individuals and across platforms. The

narrower variation in signal intensities observed for RBD-specific antibodies further supports this interpretation, indicating that quantitative measurements are less affected by methodological differences when the antigen elicits a strong and focused humoral response. On the other hand, the strong correlations for S protein may be attributed to the structural stability and immunodominant nature of Spike protein, which is the principal target of neutralizing antibodies and therefore a consistent marker across serological platforms

Overall, these correlation results underscore that while minor discrepancies exist, particularly for anti-N antibodies, the three serological platforms provide largely comparable quantitative outputs. MSD consistently demonstrated strong agreement with both ELISA and Luminex, suggesting it may serve as a robust intermediary platform when comparing datasets generated by different assay formats.

The slightly reduced correlation between ELISA and Luminex might reflect methodological differences between the two techniques, such as signal detection (colorimetric versus fluorescent) and assay dynamic range. Importantly, the high correlations observed for RBD antibodies support the use of any of the three methods in studies requiring precise quantification of neutralization-related immune responses, such as vaccine efficacy trials or longitudinal cohort studies.

Although limited by sample size and the absence of longitudinal data, these findings strongly advocate for the use of multiplex immunoassays as versatile tools for serodiagnosis.

In conclusion, multiplex immunoassays represent a major advancement in immunodiagnostics by combining high sensitivity, rapid turnaround, and the ability to measure multiple analytes simultaneously. The availability of commercial kits that allows cytokines analysis, other immune mediators, and IgG avidity assessments could further enhances their interpretative power, enabling both quantitative and functional evaluation of the immune response over time. This integrated approach offers clear clinical and public health advantages, allowing simultaneous profiling of multiple viral antigens and high-resolution immune characterization from minimal sample volumes. Such methodologies support predictive and personalized medicine, facilitate tailored clinical and vaccination strategies, improve laboratory efficiency, reduce costs, and enable rapid responses in the context of public health emergencies.

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