Deanship of Graduate Studies
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"Development of ELISA immunodiagnostic test for COVID-19 detection in infected and vaccinated human sera"

Rawand Naji Talab Ajlouni

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“Development of ELISA immunodiagnostic test for COVID-19 detection in infected and vaccinated human sera”

Prepared by:

Rawand Naji Talab Ajlouni

B. Sc in Applied Biology /

Palestine Polytechnique University / Palestine

Supervisor: Dr. Ibrahim Abbasi

Co-supervisor: Dr. Rasmi Abu Helu

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Thesis Approval

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Prepared By: Rawand Naji Talab Ajlouni

Registration number: 21911207

Supervisor: Dr. Ibrahim Abbasi.

Co-Supervisor: Dr. Rasmi abu-Helu.

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The names and signatures of the examining committee members are as follows:

1. Head of Committee: Dr. Ibrahim Abbasi signature.
2. Co-Supervisor: Dr. Rasmi Abu-Helu signature.
3. Internal Examiner: Dr. Hatem Eideh signature
4. External Examiner: Dr. Nisreen Al-Qadi signature.

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Dedication

I dedicate my thesis to my lovely parents Naji and Halah.

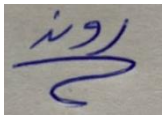
To my great husband Dr. Musab Abu Sneinh

To my family

To my teachers and friends

DECLERATION

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signature: 

Full name of the student: Rawand Naji Talab Ajlouni

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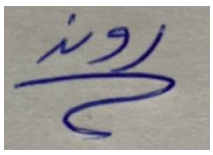
Signature: 

Abstract

Background: SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) is a novel coronavirus that has recently emerged and is causing a human pandemic. Despite the quick development of molecular diagnostic methods, validated serologic assays are necessary for detection, vaccination response study and epidemiological research. Our study's primary goal was to clone surface and membrane SARS-CoV-2 protein and use them to create an indirect ELISA so that we could screen immunity both qualitatively and quantitatively using the ELISA assay.

Methods: Membrane (M) protein and spike (S) protein of SARS-CoV-2 were cloned using Pet28-a vector in *Bl21 E. coli* bacterial cells, then ELISA assay done step by step and adjusted in the lab to test the collected samples from infected, vaccinated and non-vaccinated patients using the newly expressed protein antigens in ELISA.

Result: our study resulted in cloning of surface and membrane SARS-CoV-2 protein gene in *Bl21 E. coli* bacteria after the discovery of a point mutation. The newly cloned gene achieved a similarity of (99.3%) with sequences of SARS-CoV-2 genomes found in GenBank. The protein expression was inducted to have a crude protein with concentration of 0.5 mg\ml. The use of the recombinant S and M proteins in future screening purposes were tested using ELISA serological test, serum samples that were previously infected and then vaccinated have a higher rate of positivity (100%) using any of the two antigens, serum samples that were collected 2-3 months after vaccination have higher positive results (n= 19/25) when S antigen is used compared to M antigen (n=14/25). The number of positive results in the serum samples that collected at least 1.5 years after vaccination was 7/18 for S antigen and 9/18 for M antigen. The total samples that give positive ELISA result was 39 in S antigen and positive samples in M antigen was 36, the antibody titers related to S antigen is higher than M antigen. The titer of antibodies was higher in the samples that were previously infected and then vaccinated, then samples that were collected 2-3 months after vaccination, then lastly, the samples collected 1.5 years after vaccination.

Conclusion: The recombinant S and M antigens were cloned successfully, we recommend to use S antigen in ELISA test rather than M protein, or use them together. We recommend also to do further studies for the sensitivity and specificity, or epidemiological study.

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Abbreviations

Abbreviation	Explanation
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
ACE2	Angiotensin converting enzyme 2
CD4+	Cluster of differentiation 4
cDNA	Complementary DNA
COVID-19	Corona virus disease 2019
CRISPR-CasN	Clustered regularly interspaced short palindromic repeats
CT	Chest computed tomography
DNA	Deoxyribonucleic acid
DsRNA	Double-stranded RNA
E. coli	Escherichia coli
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked-immunosorbent assay
FCS	Fetal calf serum
FDA	Food drug association
FET	Field-effect transistor
HRP	Horseradish peroxidase
IFN	Interferons
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ILFA	Immunochromatographic lateral flow assays
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
KDa	Kilo-dalton
LAMP	Loop-mediated isothermal amplification
LB broth	Luria-Bertani broth

M protein	Membrane protein
MCDA	Multiple cross displacement amplification
MERS	Middle east respiratory syndrome
NAAT	Nucleic acid amplification tests
NEAR	Nicking and extension amplification reaction
NP	Nucleocapsid protein
OD	Optical density
ORF	Open reading frame
PBS	Phosphate-buffered saline
PBS-T	Phosphate buffer saline-tween 20
PCR	Polymerase chain reaction
PH	Potential of hydrogen
R0	Reproduction number
RBD	Receptor binding domain
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RPA	Recombinase-aided amplification
RT	Reverse transcriptase
RTIs	Respiratory tract infections
RT-PCR	Real time polymerase chain reaction
S protein	Spike protein
SARS CoV2	Sever acute respiratory syndrome Coronavirus 2
SPR based biosensor	Surface plasmon resonance biosensor
SsRNA	Single-stranded RNA
TAE buffer	Tris-acetate-EDTA
TBST	Tris-buffered saline and Tween 20
Th1	T helper cell type 1

1.Introduction

1.1 Introduction

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which infect the respiratory tract causing acute respiratory tract infection (ARTI). Originated in Wuhan in December 2019, China (Hui et al.,2020). Mild to moderate respiratory illness are experienced by most people infected with the virus and usually don't need any special treatment. However, some will become seriously ill with symptoms like pneumonia, fever, cough and respiratory distress and those require special medical care. Older adults and people with existing persistent health problems are more likely to present with more severe disease than people of all ages (Wang et al.,2020).

The studies shows that the virus may spread by Symptomatic, asymptomatic and pre-symptomatic patients and because of the majority of COVID-19 patients have a moderate or undetectable illness (with no symptoms), half of all COVID-19 cases are infected through asymptomatic transmissions (Tabata et al.,2020). The incubation time for COVID-19 varies widely among patient populations, and as symptoms might occur between two and fourteen days after infection, viral transmission may go undetected (Long et al., 2020).

Thus fast, early and accurate tests to diagnose COVID-19 disease is important for chasing SARS-CoV-2 transmission, the control of the transmission of the virus, investigations into the epidemiology, the supervision of each individual case, and carrying out of quarantine laws in order to prevent new cases and fatalities.

Testing technologies based on Nucleic-acid detection that use (RT-PCR) to detect the virus still the best diagnostic test available, because it is highly sensitive and very accurate test to detect COVID-19 patients in the early stage of the disease (Pan, Y et al.,2020) RT-PCR detect the SARS-CoV-2 RNA genome in nasal and throat swaps of infected persons (Corman et al., 2020).

As noted, the amount of virus found in COVID-19 patients' upper respiratory tract secretions peaks seven days after the onset of symptoms, but later in the course of the illness, it may decline

greatly below the threshold of RT-PCR detection. Additionally, failing to observe the viral replication time window will produce erroneous negative results (Pan et al., 2020).

Thus, in order to ensure the early diagnosis of all COVID-19 patients, additional screening assay approaches for the detection of SARS-CoV-2 are urgently required. Assessment of specific antibodies against SARS-CoV-2 offers an alternative very sensitive and accurate diagnostic method that may make up for the drawbacks of RT-PCR including the need for expensive thermocycler and professional staff to perform the test and interpret results, the standard control has an important role in the accuracy of the results and the problem of obtaining false-negative results due to time and quality of sample collection, sample degradation and the low efficiency of some test kits. Computerized tomography scan system (CT) is expensive technique, require technical expertise, and results interpretations would be to other diseases than COVID-19 (Sheikhzadeh et al., 2020).

Immunoglobulin M (IgM), G (IgG), and A (IgA) generated in patient body shortly post-disease onset. Serological assays can enable serological monitoring, identify individuals who have already been infected, and monitor the COVID-19 outbreak's spread. Moreover, serological assays may make it easier to assess the evaluation of immunity generated by vaccines and identify suitable convalescent plasma donors (Føns et al., 2021).

In contrast to molecular assays, such as RT-PCR, serological assays for the diagnosis of SARS-CoV-2 are frequently quicker, less expensive, and simpler to carry out by staff without considerable laboratory qualifications. The ability to detect the production of antibodies may be a tool that, either by itself or in combination with PCR, improves the sensitivity and accuracy of detection. The FDA (Food Drug Association) has approved a number of commercial serological kits for assessing SARS-CoV-2 IgG, IgA, and IgM antibodies (Younes et al., 2020).These tests specifically target the N protein, S protein, S1 fragment, and receptor-binding domain (RBD) which is the of immunogenic proteins of coronaviruses (Sheikhzadeh et al., 2020).

Enzyme-linked immunosorbent assay (ELISAs) one of the most widespread serological assays used in COVID-19 diagnosis , sensitive method to detect the antigen or antibody of interest in the samples , could be in direct or indirect formats, direct ELISA , an enzyme-linked antibody directly binds to the antigen in the sample where as in indirect ELISA , a primary antibody is used to bind to an antigen which was coated on a microplate , Then secondary enzyme-labeled antibody is

applied to detect the primary antibody , the binding reveal positive ELISA test (Sheikhzadeh et al., 2020).

However, the kind of viral protein utilized as a capture antigen determines the sensitivity and specificity of enzyme-linked immunosorbent tests (ELISAs). Additionally, according to patient diversity, disease severity, and immunological status, antibody seroconversion in COVID-19 patients might happen fairly late after disease onset. Specific antibodies against SARS-CoV-2 are produced by the majority of COVID-19 patients after seven to eleven days of exposure, with considerable disagreements have been documented (Lagousi et al., 2019) which leads to a challenge of overcoming this diversity and obtain specific accurate test results.

In this study, we aim to clone spike (S) and (M) protein of SARS-CoV-2 in high amount as capture antigen so we can develop ELISA test to readily define a true positive SARS-CoV-2 infection with high specificity and sensitivity and study the efficiency of the vaccines.

1.2 Problem statement.

Current COVID-19 diagnostic tests rely on molecular approaches like RT-PCR which provide good sensitivity and specificity and the results can be obtained in a few hours. It can detect viral DNA in respiratory samples, saliva, blood, urine and stool. However, RT-PCR has some drawbacks including the need for expensive thermocycler and professional staff to perform the test and interpret results, the standard control has an important role in the accuracy of the results and the problem of obtaining false-negative results due to time and quality of sample collection, sample degradation and the low efficiency of some test kits. Computerized tomography scan system (CT) is expensive technique, require technical expertise, and results interpretations would be to other diseases than COVID-19 (Sheikhzadeh et al., 2020).

Thus, the need to develop a complementary cost effective, easy, and fast diagnostic assay in Palestine is arising.

ELSA technique is easy to perform, can detect serum antibody which will help also in investigating the immunity of patients, vaccine response and herd immunity. So, in this study we are going to clone spike and membrane protein of the virus as they are surface protein to use them in developing ELISA to detect infected and vaccinated individuals.

1.3 Study significance.

COVID-19 pandemic is a dangerous serious global health issue today due to the rapid human to human transmission of SARS-CoV-2 311 million cases around the world and more than 473 thousand registered cases in Palestine, COVID-19 disease could be fatal, more than 5.5 million deaths worldwide and approximately 5000 deaths in Palestine based on the World Health Organization's official daily COVID-19 Situation Reports according to World Health Organization. Coronavirus Disease (COVID-19) Outbreak Situation. Available online, accessed on 11 June 2022 (World Health Organization, 2022).

SARS -CoV-2 has a faster rate of transmission than other coronaviruses such as SARS and MERS and until now there are no approved specific drugs or vaccines for treatment. Thus, early diagnosis is crucial to prevent the extensive spread of the disease.

Spike and membrane protein was cloned to develop an indirect ELISA to detect IgG antibodies against SARS-CoV-2; to detect the late stage in the patients when the virus is undetectable by PCR or study the immune response to the vaccines. So, ELISA is fast and cheap method to relay on, especially in Palestine where it's worth it financially to make this study.

1.4 Aims and objectives

The major aim of this study is to clone COVID-19 proteins to be used in serological tests.

1. To amplify the spike and membrane protein of SARS-CoV-2 by specific primers.
2. To clone S and M SARS-CoV-2 antigens by inserting the gen of each into (pET 28a) vector and then transform into (*E. coli* strain *BL21*) host cell to express the protein of interest heavily.
3. To use the expressed proteins in developing immunoassay test for SARS-CoV-2.
4. To validate the developed ELISA in the detection of infected and vaccinated persons.

2. Literature review

2.1 History

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which infect the respiratory tract causing acute respiratory tract infection (ARTI). Originated in Wuhan in December 2019, China (Hui et al., 2022).

According to the World Health Organization's official daily COVID-19 Situation Reports, on December 22 there were over 672 million cases and over 6 million fatalities reported globally, with around 703 thousand cases and 5 thousand deaths in Palestine. Situation with the COVID-19 coronavirus outbreak, accessible online, accessed on 11 February 2023 (World Health Organization,2022). Most infected persons show mild to severe respiratory illness and don't require any particular care. But some people will get serious illnesses that need for specialized medical attention in hospital, such as pneumonia, coughing, fever, and respiratory distress. Older adults and people with underlying chronic medical issues are more likely to present with more severe disease than people of all ages (Wang et al.,2020).

The studies shows that SARS-CoV-2may spread by Symptomatic, asymptomatic and pre-symptomatic patients (He et al., 2020). Additionally, since the majority of COVID-19 patients have minor or no symptoms, asymptomatic transmissions account for 50% of all COVID-19 cases. (Tabata, S et al., 2020) The incubation time for COVID-19 varies widely among patient populations, and as symptoms might occur 2–14 days after being exposed, viral transmission may go undetected (Long et al., 2020).

Back to 2002, The first known cases of Severe acute respiratory syndrome (SARS) occurred in November 2002 by the spread of SARS-CoV-1 , and the syndrome caused the 2002–2004 SARS outbreak (Li et al., 2020) .Infected over 8,000 people from 29 countries and territories, and resulted in at least 774 deaths worldwide (Live et al., 2002).

Flu-like symptoms associated with SARS-CoV-1 include fever, muscle aches, fatigue, coughing, sore throats, and other general symptoms. A temperature that is higher than 38 °C (100 °F) appears

to be the only symptom shared by all individuals. Breathing difficulties and pneumonia, either primary viral pneumonia or subsequent bacterial pneumonia, are frequent side effects of SARS-CoV-1. SARS-CoV-1 typically takes 4-6 days to incubate, while it hardly ever takes just 1 day or as long as 14 days (World Health Organization, 2023).

There is another coronavirus related disease that immerged in 2012, Middle East respiratory syndrome (MERS) is a viral respiratory infection caused by Middle East respiratory syndrome–related coronavirus (MERS-CoV). Symptoms can vary depending on age and risk level from none to mild to severe. Fever, cough, diarrhea, and shortness of breath are typical symptoms. People with significant health issues often experience a more severe case of the condition (Zumla et al., 2015).

2.2 Phylogenetics and Taxonomy.

SARS-CoV2 is a member of the broad family of viruses known as coronaviruses, after 229E, NL63, OC43, HKU1, MERS-CoV, and SARS-CoV1; SARS-CoV-2 is the seventh coronavirus known to infect humans (Guan et al., 2020). SARS-CoV-2 is a member of the subgenus *Sarbecovirus* (*beta-CoV* lineage B), see figure (1) bellow .

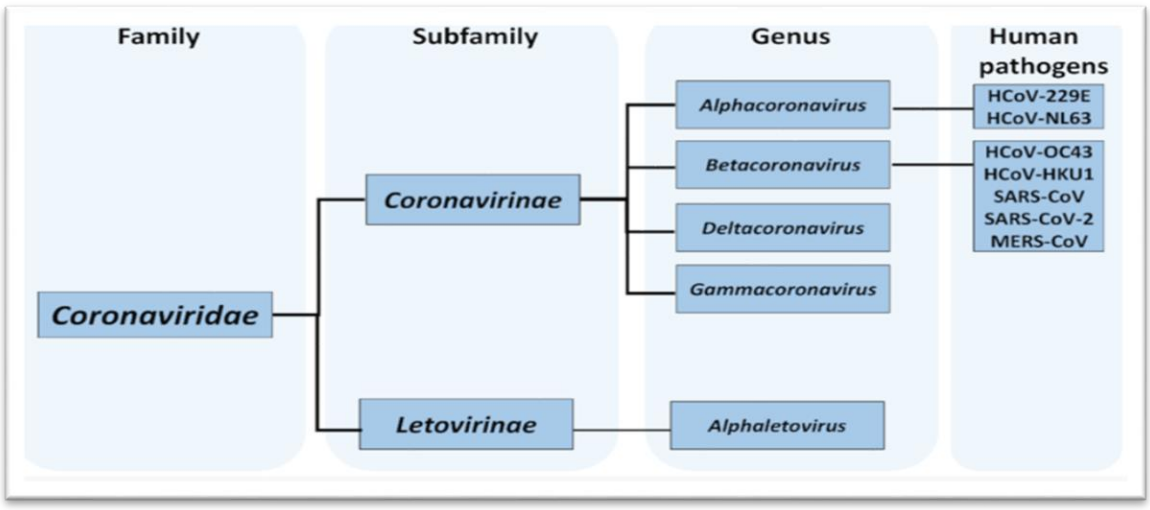

Figure 1: Taxonomy of Coronaviridae family with an indication of species known to be pathogenic to humans and cause respiratory diseases (International Committee on Taxonomy of Viruses (2022)).

Several notable variants of SARS-CoV-2 emerged in late 2020. The World Health Organization has currently declared five variants of concern, which are as follows: Alpha: In September 2020,

Lineage B.1.1.7 first appeared in the United Kingdom, showing signs of heightened virulence and transmissibility. The mutations N501Y and P681H are notable examples. Several public health organizations have reported and are monitoring an E484K mutation in some lineage B.1.1.7 viruses. Beta: In May 2020, the lineage B.1.351 virus first appeared in South Africa. Evidence of greater transmissibility and antigenic alterations was found, and some public health professionals raised concerns about it's potential impact on the effectiveness of various vaccines. The mutations K417N, E484K, and N501Y are notable examples. Gamma: Lineage P.1 first appeared in Brazil in November 2020, along with signs of altered antigenicity, increased transmissibility, and virulence. Similar issues with vaccine effectiveness have been brought up. Additionally notable alterations are K417N, E484K, and N501Y. Delta: In October 2020, Lineage B.1.617.2 first appeared in India. Additionally, there is proof of altered antigenicity and increased transmissibility. and finally ,Omicron: Lineage B.1.1.529 emerged in Botswana in November 2021 (Prüβ and B. M,2022) .

2.4 Biology.

2.4.1 structure and genome.

SARS-CoV-2 virion diameter's is 60–140 nanometers (Remitente et al., 2020). The four structural proteins found in SARS-CoV-2 are known as the S (spike), E (envelope), M (membrane), and N (nucleocapsid); the N protein protects the RNA genome, while the S, E, and M proteins work together to form the viral envelope. Glycoproteins and type I membrane proteins both make up the coronavirus S proteins (membranes containing a single transmembrane domain oriented on the extracellular side) (Wrapp et al., 2020). S protein divided into two parts (S1 and S2), it's the protein that allows SARS-CoV-2 to replicate, it facilitates the virus's attachment to and fusion with a host cell's membrane; more precisely, its S1 subunit catalyzes attachment and the S2 subunit fusion (Wrapp et al., 2020).

SARS-CoV-2 RNA genome is linear, positive-sense, single-stranded, about 30,000 bases long. The SARS-CoV-2 genomes encode five basic open reading frames (ORFs), including four canonical 3′ structural proteins that are shared by all coronaviruses: the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins, and 5′ frameshifted polyprotein (ORF1a/ORF1ab) (Ashour et al., 2020).

In addition, a number of accessory genes unique to particular subgroups are discovered scattered throughout or even overlapping the structural genes. Overprinting is a process that causes

the expression of new proteins in alternate frames as a result of nucleotide changes in a pre-existing frame. It is believed that the accessory proteins in coronaviruses have additional functions that are typically implicated in pathogenicity in the natural host but are not always necessary for viral replication. The quantity, location, and size of the accessory proteins in coronaviruses differ between the various viral subgroups (Schaecher et al., 2010).

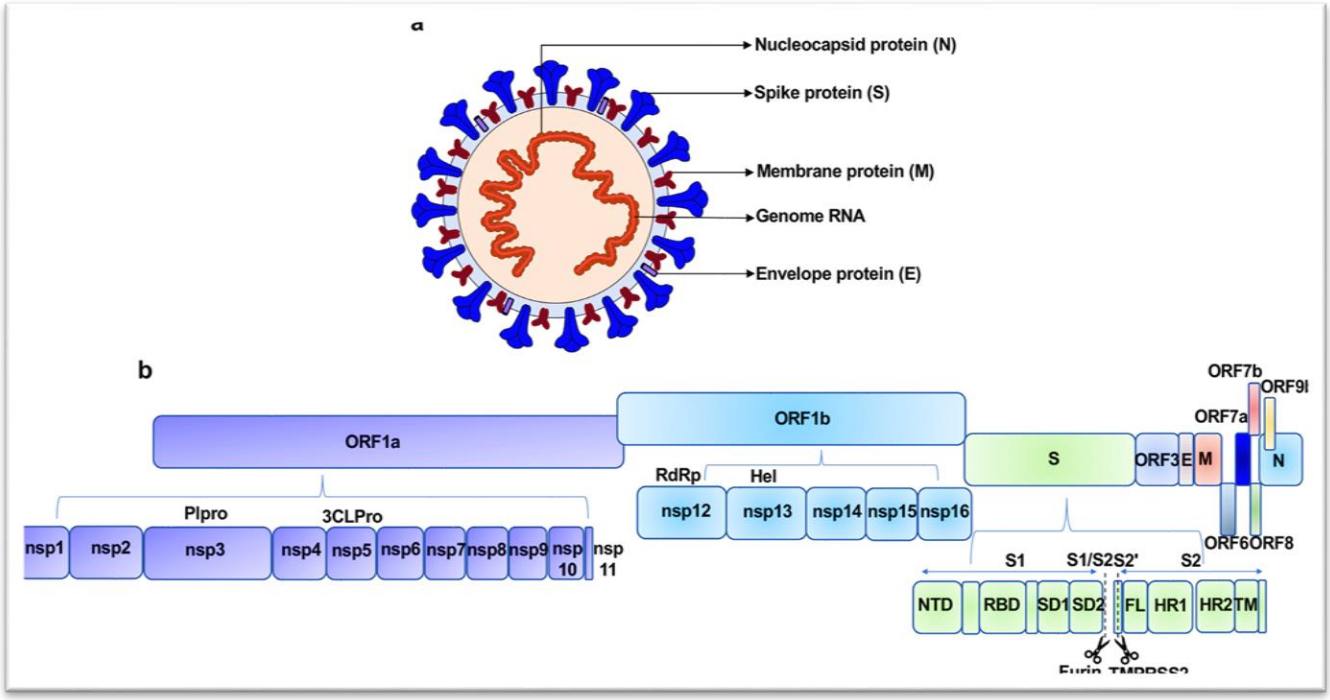

Figure 2: Structural proteins of SARS-CoV-2 including Spike protein (S), Membrane protein (M),Envelope protein (E) and Nucleocaspid protein (N) (Zhang et al., 2021).

Less research has been done on accessory proteins for two main reasons. while accessory proteins are typically not required for viral replication or structure, they might influence the host's interferon signaling pathways and contribute to viral pathogenicity or spread (Michel et al., 2020). Figure2 illustrates the SARS-CoV-2 structure and gen. The genome includes ORF1a-ORF1b-S-ORF3-E-M-ORF6-ORF7 (7a and 7b)-ORF8-ORF9b-N in series, see figure (2) (Zhang et al., 2021).

2.4.2 Life cycle and Pathogenicity.

The spike (S) protein of coronaviruses interacts to cellular receptors that responsible for entrance, including: human aminopeptidase N, angiotensin-converting enzyme 2 and dipeptidyl peptidase 4. As a result, viral tropism and pathogenicity are influenced by the expression and tissue distribution of entrance receptors (V'kovski et al., 2021).

The infection starts by interaction between the spike glycoprotein (receptor binding domain) on the envelope of the virus with one of the body receptors ACE2. SARS Cov2 and SARS Cov1 share 70% genome similarity, the main differences between them that the affinity of the receptor-binding domain (RBD) is 10 fold higher than SARS CoV1 which give more availability of cell entry, another feature that SARS CoV1 always show standing up RBD to be ready for binding ACE2, while SARS CoV2 is choosing the lying state of the RBD by hiding it in the spike protein ; thus provides an evasion mechanism of covid19 from the neutralizing antibodies, it also manipulate other immunological processes including antigen presentation and cytokines (Taefehshokr et al., 2020).

SARS-CoV2 has multiple steps in its life cycle inside the host cell. In a summary, it starts with the virus's Spike-Fc protein attaching to the angiotensin-converting enzyme-2 (ACE2) receptor on bronchial epithelial cells, alveolar pneumocytes. As a result, ACE2 expression is downregulated, resulting in severe acute respiratory failure (Khade et al., 2021). In addition to the ACE2 receptor protein being expressed on the surface of lung epithelial cells, ACE2 protein is also expressed on the surface of small intestine epithelial cells, suggesting that SARS-CoV2 pathogenesis could take another pathway. The endocytosis process begins when a ligand binds to the receptor and the membrane fuses, allowing virions to enter the host cell. The virion's genetic material is released into the host as a result of the membrane fusion. The viral RNA is translated into the functional RNA Polymerase protein by controlling the host ribosome machinery. The positive ssRNA is replicated into dsRNA by the expressed viral RNA polymerase. Nucleocapsid (N), membrane (M), spike (S), and envelope (E) proteins RNA transcription at the sub-genomic level. N protein translation takes place in the cytoplasm, whereas S, M, and E protein translation takes place in the rough endoplasmic reticulum (RER) due to post-translation changes. The structural proteins S, M, and E assemble with the viral nucleocapsid (N). In the Golgi vesicle, the assembled viral components are further matured to create the mature virion with the lipid envelope. Exocytosis is

the process by which mature virions are discharged into the environment (Khade et al., 2021). See figure (3) (Harrison et al., 2020).

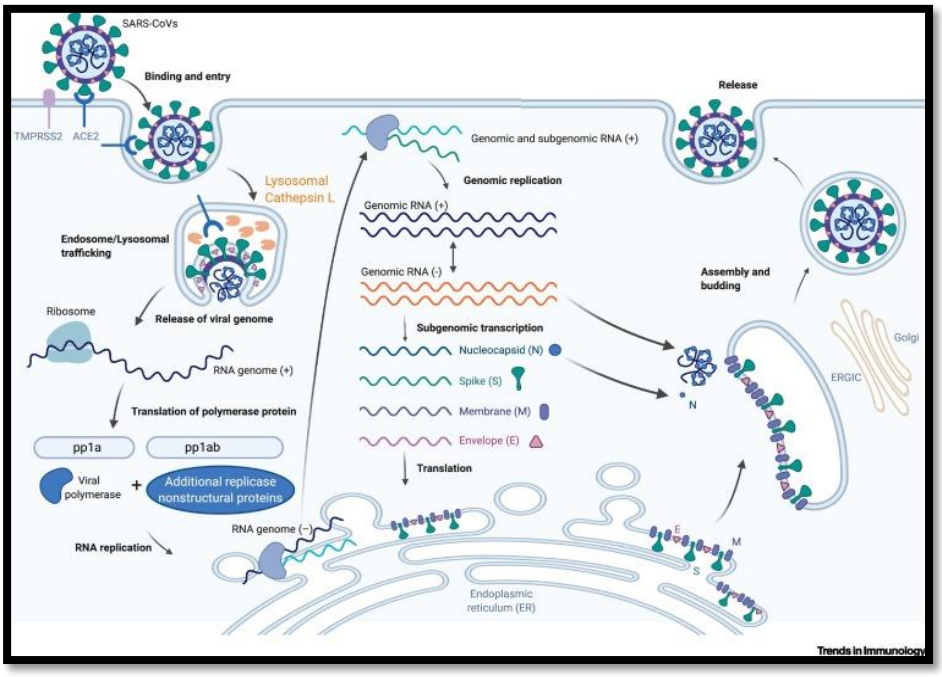

Figure 3: The lifecycle of SARS CoV-2 (Harrison et al., 2020).

2.5 Immunity to SARS -CoV- 2.

The host immune system detects the entire virus or its surface epitopes once the virus has entered the target cell, triggering an innate or adaptive immune response. Toll-like receptors 3, 7, and 8 on immune cells, in particular, are the first pathogen recognition receptors (PRRs) to recognize the virus, which increases interferon (IFN) production. During SARS-CoV1 and MERS-CoV infection, their non-structural proteins impede the activity of host innate immune cells, which alters the overall release of cytokines (Shah et al., 2020).

It has been discovered that the humoral response to SARS-CoV-2 is comparable to that against other coronavirus infections, involving the generation of the typical IgG and IgM. When SARS-CoV1 infection first occurs, B cells produce antibodies to the N protein right away, whereas antibodies to the S protein can only be found 4 to 8 days after the onset of the first symptoms. (Shah et al., 2020). After the beginning of symptoms, SARS-CoV2-specific IgA, IgG, and IgM

antibodies were found in infected individuals at various times. IgG was shown to be present for a longer amount of time than IgM, which began to drop after three months (Shah et al., 2020).

According to the fundamental principles of immunology, the immune cell response to viral infections is known to play a significant role in preventing clinical development and providing protection against recurrent infections. which also relates the SARS-CoV-2 illness. In fact, COVID-19 was discovered to be effectively regulated in the majority of infected persons by the coordinated activation of the innate and adaptive immune components (Sette et al., 2020).

people recovering from SARS-CoV-2 infection up to 18 months after infection, the existence of CD4+ and CD8+ T-lymphocytes has been shown throughout time, according to a few recent reports. Additionally, it was demonstrated that this T-lymphocyte-based immunity existed regardless of how severe the clinical picture connected to the infection itself was (Breton et al., 2021).

On the other hand, these viruses may also suppress anti-viral IFN responses by eluding the innate immune cells. Pro-inflammatory cytokines are produced in greater amounts as a result of the infiltration of neutrophils, monocytes/macrophages, and other adaptive immune cells. Th1/Th17 cell activation with viral epitopes in the helper T cell fraction may intensify inflammatory reactions as cytokine which will result in immunopathologies such pneumonia and pulmonary edema. Infected cells in the lungs are targeted for destruction by cytotoxic T lymphocytes sent to the infection site. In addition to recognizing viral proteins, B cells and plasma cells are activated to release antibodies that are specific to SARS-CoV-2, which may promote in the destruction of viruses and offer systemic immunity in several organs (Shah et al., 2020).

For immunity after vaccination، after the doses of the vaccine are given, the antibody titers start to decline quite quickly. Such losses occur more quickly than the induced protection against serious disease (De Bruyn et al., 2022). The effectiveness against COVID-19 infections with symptoms was observed to rapidly degrade in 842,974 vaccinated individuals after roughly 6-7 months, and may even become negative for longer time periods (Redd et al., 2021).

The authors have proposed that the ongoing development of novel variations could be responsible for the vaccination's short-term effectiveness. Additionally, vaccines are known to trigger an immune response that targets the spike protein; in fact, the majority of virus mutations only impact this protein, which may further help to explain why vaccines only have a temporary protective effect (Diani et al., 2022).

2.6 Antibodies profile in COVID-19 patients.

An indirect enzyme-linked immunosorbent assay (ELISA) was used to examine serial specific IgA, IgM, IgG, and IgG isotypes including IgG1 to IgG4 responses using four recombinant SARS-CoV-2 antigens (including receptor binding domain (RBD), S1 protein, nucleocapsid protein (NP) and ectodomain of spike protein). The results of this retrospective study were published in 2020 by Yin and Tong et al, IgG2 and IgG4 levels were demonstrated to be undetectable. IgG levels against all four antigens peaked 27 days after the onset of symptoms and then gradually decreased till 91 days later (Yin et al., 2020).

IgG1 against ECD, S1, and RBD peaked at 7 days after the onset of symptoms, while it peaked at 14 days after symptoms onset when it tested against NP. All of these IgG1 levels were high up until the 91st day after the onset of symptoms. IgG3 responses to S1, ECD and NP were elevated starting on the fourth day and gradually decreased until the 91st day. Beginning on day 4, IgA levels that responded to ECD and RBD increased and remained elevated until day 91. On the contrary, IgA that was tested specifically against S1 and NP decreased rapidly after reaching a peak and was undetectable by the 91st day after the onset of symptoms. Additionally, from the seventh day to the seventeenth day, IgM that responded with NP significantly reduced and remained undetectable, see figure (4) (Yin et al., 2020).

In general, Following SARS-CoV-2 infection, IgM and IgG antibody production tends to peak 11 to 14 days after the onset of symptoms, earlier than in other viral infections. IgM and IgG antibodies sometimes appear immediately, in contrast to many other viral infections, where IgM antibodies frequently appear several weeks before IgG antibodies (Peeling et al., 2022).

2.7 Epidemiology of COVD-19.

2.7.1 Epidemiology of COVD-19 worldwide.

Droplet transmission of SARS-CoV-2 occurs when a person comes into close contact

(Less than 1 meter) with someone who is coughing or sneezing. droplets from the infected individual's mucosae (mouth and nose) or conjunctiva can potentially transfer the virus (eyes). Although mucosal transmission is the most common, viral transmission through the conjunctiva is rather uncommon (Zhou et al., 2020).

SARS-CoV-2 appears to have a less fatality rate than SARS-CoV1 or MERS CoV, despite the large number of deaths related with COVID-19. Because of the rapid spread of disease, public health officials and government agencies have implemented unprecedented measures such as travel.

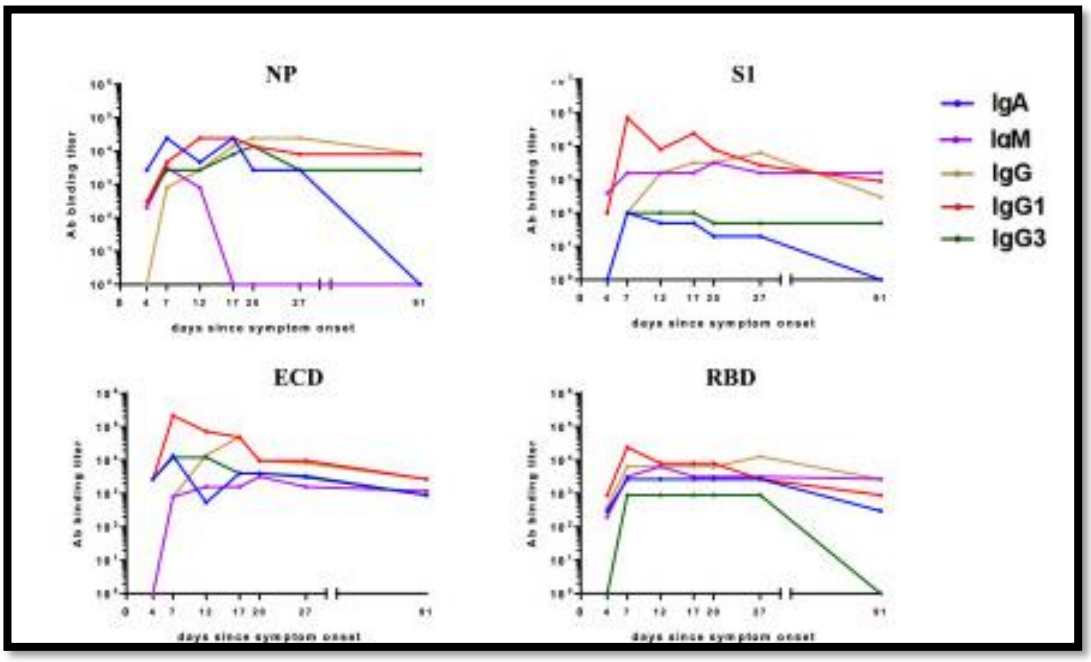

Figure 4: IgM, IgA, IgG, IgG1 and IgG3 antibody titers in response to SARS-CoV-2 antigens (Yin et al., 2020).

According to a meta-analysis dated November 2020, the virus's basic reproduction number (display style R 0 R 0) is estimated to be between 2.39 and 3.44 which mean each virus infection could cause 2.39 to 3.44 additional infections if no one in the community has an immunity to it and no preventative actions are done, more dense areas tend to have higher R0. Since human behavior influences the R0 value, estimates of R0 vary among nations, cultures, and social rules. For instance, one study discovered that Sweden, Belgium, and the Netherlands had relatively low

R0 values (3.5), whereas Spain and the US had much higher R0 values (5.9 to 6.4, respectively) See figure 5 (Ke et al., 2021). See figure 5.

In mainland China, there have been around 96,000 confirmed cases of illness. While the percentage of infections that lead to confirmed cases or develop into diagnosable diseases is yet unknown, one mathematical model estimated that 75,815 persons were infected on January 25, 2020 in Wuhan alone, even though there were only 2,015 confirmed cases globally. In Hubei province, where Wuhan is situated, more than 95% of all COVID-19 deaths that happened globally before February 24, 2020, had taken place. As of February 9, 2023, the proportion was 0.047%, there have been 672,273,129 total confirmed cases of COVID-19 in the ongoing pandemic. The total number of deaths attributed to the virus is 6,848,748 (Dong et al., 2022).

2.7.2 Epidemiology of COVD-19 in Palestine.

On March 4, 2020**,** seven Palestinians tested positive for SARS-CoV-2 in Bethlehem, after that, the Palestinian government declared a state of emergency lasting one month on March 5, 2020. People were quarantined unless in emergency cases after a state of emergency was declared. The state of emergency has been declared in effect for one more month. (Ministry of Health, State of Palestine,2023).

Due to a decline in incidences and an increase in the rate of positive tests among Palestinian laborers leaving Israeli land, the limitations were lifted on May 25th, 2020. In July 2020, the number of cases rose once more, with Hebron serving as the heart of the outbreak and housing more than 70% of all active cases. The West Bank was placed under a 15-day total lockdown beginning on July 3, 2020 (Ministry of Health, State of Palestine,2023).

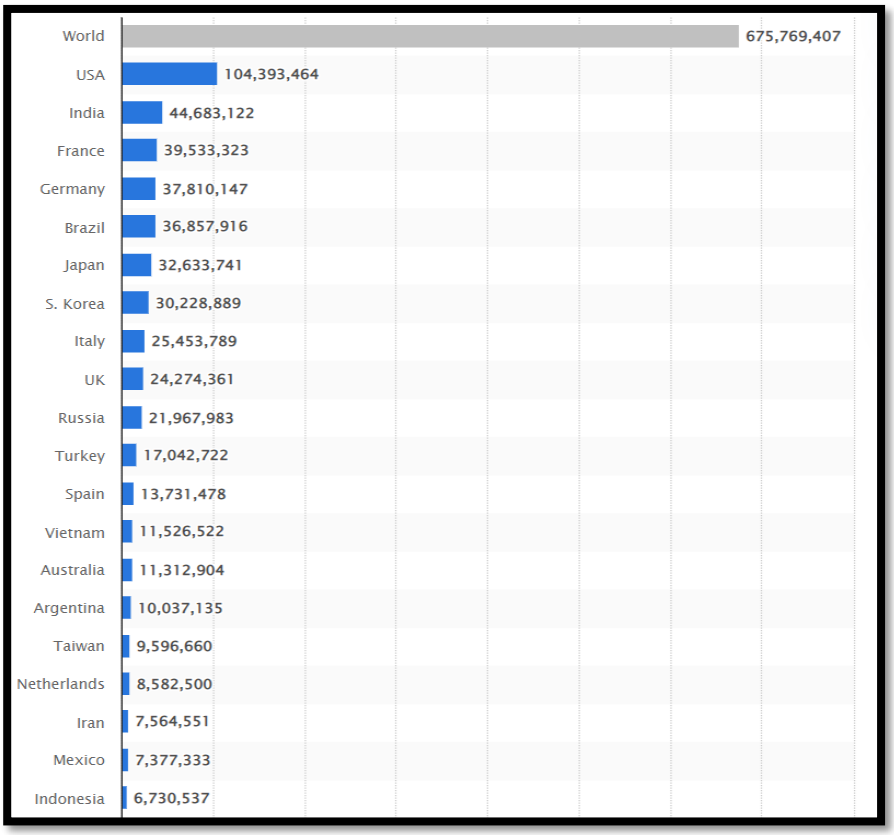

Figure 5: COVID-19 cases worldwide as of February 3, 2023, by country or territory (Statista ,novel-coronavirus statistics, 2019).

On July 12, 2020, a five-day total lockdown was imposed on the governorates of Hebron, Bethlehem, Ramallah, and Nablus. People were subject to night and weekend curfews, with the exception of a few approved services, and movement between governorates was prohibited until July 27, 2020. It was forbidden to travel between governorates or attend any social events in public. After July 13th, 2020, the Palestinian administration announced a relaxation of the restrictions, enabling small enterprises to reopen with limits and commercial travel across governorates. (Ministry of Health, State of Palestine,2023).

The Palestinian Ministry of Health recorded 210 073 cases and 2275 deaths as of March 1st, 2021. However, because the majority of people had been infected the government opened the nation's roads, lifted the restrictions, and administrating vaccines (Coronavirus (COVID-19) in Palestine,2023.

And finally, by February 2023 Data, Palestine recorded 703228 Coronavirus Cases since the epidemic began, according to the World Health Organization (WHO). In addition, Palestine reported 5708 Coronavirus Deaths. See figure 6 (World Health Organization, 2023).

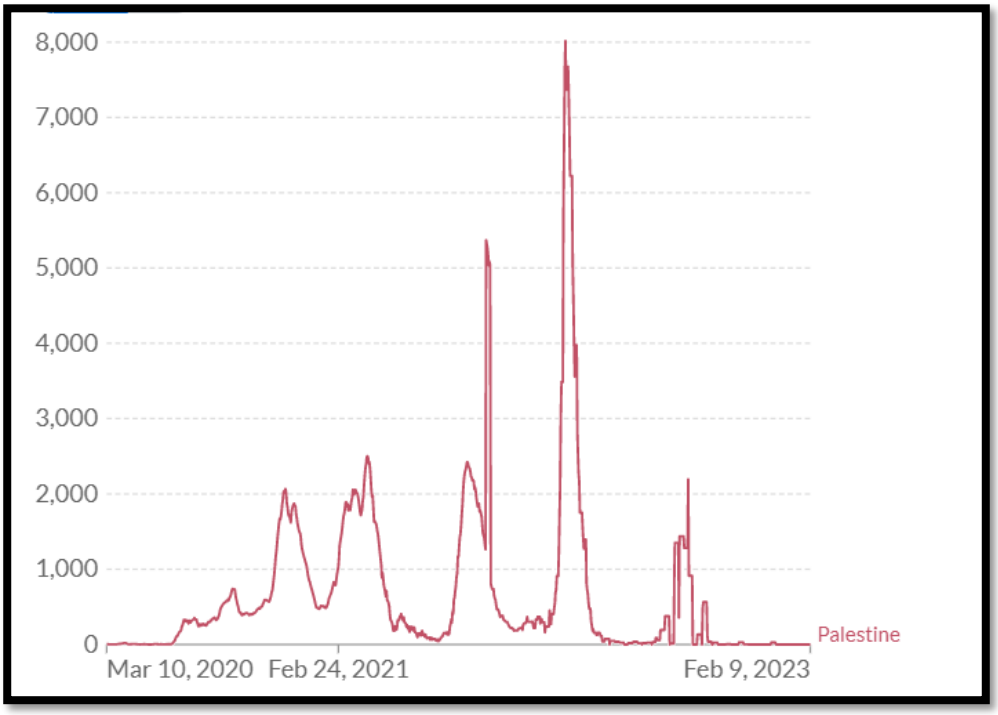

Figure 6: This chart shows the number of confirmed COVID-19 cases per day in Palestine **(**Coronavirus Recourses center**,** Johns Hopkins University CSSE COVID-19, 2023).

2.8 Diagnosis of SARS Covid 2.

2.8.1 Molecular Diagnosis.

Clinical diagnosis of COVID-19 has been basically been based on signs and symptoms evaluation, and confirmed by nucleic acid amplification tests (NAAT), such as, RT-PCR (Reverse Transcription Polymerase Chain Reaction) of nasopharyngeal or oropharyngeal swabs. The reverse transcription of coronavirus ribonucleic acid (RNA) into complementary DNA (cDNA) serves as the starting point for these assays, which are utilized to amplify small amounts of deoxyribonucleic acid (DNA). Then, PCR is carried out, and the resulting DNA amplification is subjected to precise detection using various analytical techniques (Kashir et al., 2020). then quantifying viral load using the cycle threshold (Ct); The number of cycles needed for the fluorescent signal to exceed the background level and cross the threshold. The amount of target

nucleic acid in a sample is inversely correlated with the Ct level; the lower the Ct level, the higher the amount of target nucleic acid in the sample. The genomic regions of SARS-CoV-2 that code for the proteins spike, nucleocapsid, and enveloped were used to make particular primers and probes (Sharma et al., 2021).

Loop-mediated isothermal amplification (LAMP), multiple cross displacement amplification (MCDA), recombinase-aided amplification (RPA), nicking and extension amplification reaction (NEAR), and CRISPR-CasN-based assays are all among of the amplification types included in NAAT (Zhang et al., 2020).

Although techniques that based on PCR are simple, have high sensitivity, and extremely specific, and they can regularly and consistently identify coronavirus infection in individuals, still this technique has some drawbacks, like needing expensive specialized equipment and highly trained analysts and technicians. moreover, PCR have high false-negative rate results and requires 4–8 h to process the samples and 1–3 days to report the results (Maia et al., 2022).

Reliable and quick serological diagnostic approaches are critically needed to screen COVID-19 patients and those without evident symptoms or who passed 12 days on their onset of infection, Serological tests, whether they take the form of ELISA or immunochromatographic lateral flow assays (ILFA), are currently the subject of a lot of research. Due to the SARS-CoV-2 infection, viral protein antigens and antibodies like IgM, IgG, IgA are produced into the serum of the patient, which can be targeted and can offer a wider time window for indirect detection of SARS-CoV-2 (Zhang et al., 2020).

2.8.2 Serological Diagnosis.

Serological tests are diagnostic methods that are used to identify antibodies and antigens in a patient's sample The presence of antibodies against a pathogen in a person's blood indicates that they have been exposed to that pathogen. Most serologic tests measure one of two types of antibodies: immunoglobulin M (IgM) and immunoglobulin G (IgG). IgM is produced in high quantities shortly after a person is exposed to the pathogen, and production declines quickly thereafter. IgG is also produced on the first exposure, but not as quickly as IgM. On subsequent

exposures, the antibodies produced are primarily IgG, and they remain in circulation for a prolonged period of time (Turgeon and M. L, 2015).

This has an impact on how serology results are interpreted because a positive IgM result indicates that a person is currently or recently infected, but a positive IgG result and a negative IgM result indicate that the individual may have previously been infected or immunized. Antibody testing for infectious disorders is frequently conducted twice: once during the acute stage of the illness and once again after recovery (convalescent phase). When the antibody titers of the two samples are examined, the convalescent specimen's much greater IgG level confirms infection rather than prior exposure. People who are immunosuppressed, since they make less antibodies, and those who take antimicrobial medications early in life may have false negative findings for antibody tests (Berkowitz et al., 2016).

Based on antibody detection, serological testing can be conducted using lateral flow immunochromatographic analyses, lateral flow immunochromatographic strips, chemiluminescence immunological assays and enzyme-linked immunosorbent assays (ELISA). Now, there have been a lot of studies concerned about commercial antigen detection kits for SARS-CoV-2 (Lambert-Niclot et al., 2020).

Although these serological assays aren't very good at identifying acute infections, they do allow for a number of crucial uses. First, serological assays allow to explore the immune responses to SARSCoV-2 in a qualitative and quantitative way. Serosurveys are necessary to accurately assess the infection rate in an affected region, which is a crucial factor in figuring out the infection mortality rate. serological testing will make it possible to find persons who have strong antibody reactions and may be recruited as donors for the production of convalescent serum treatments Serological tests may also be used to distinguish between those who are immune and those who are not. These details might be extremely helpful for carefully placing immune healthcare workers to lower the risk of exposure and inadvertent virus spread. Additionally, it might enable a portion of the populace to resume "normal life" after having built up immunity. Parallel investigations determining whether antibody titers correlate with protection are necessary to support these prospective measurements (Stadlbauer et al., 2020).

ELISA based antigen SARS CoV detection in different nasopharyngeal aspirate, urine, and fecal specimens was the new suggested mechanism with better advantages than previous ones, like cheaper, no need for intense labor, easy to use, no contamination, and high specific technique. Serology tests complementary to molecular methods for diagnosis COVID-19 (e.g.,diagnosis of asymptomatic patients) and epidemiological claims. In the present time of COVID-19 vaccination, serological testing will be widely used to estimate the vaccine efficiency (Alandijany et al., 2021).

A study published in the literature used antibody detection to test the source of the immune response in humans. The study used ELISA to detect antibodies against the N and S proteins, and the results showed 100% sensitivity which mean no false negatives, 98.9% specificity minimal false positives, and zero cross-reactivity with other coronavirus antibodies. S protein-based ELISA and N protein-based ELISA were used to examine the specificity of the target N protein antibody. The results showed that both approaches were positive for infected individuals and that only S protein-based ELISA was positive for individuals who had received the vaccination. Sensitivity of 12 all positive test findings evaluated by micro-neutralization assay, then tested on the N protein ELISA which gave all positive results 100% (Alandijany et al., 2021).

S and N proteins have attracted the most focus in the identification of the SARS-CoV-2 virus to date. In addition to the S protein, the M protein was the focus of this study since it is the most prevalent structural protein of the SARS-CoV-2 and serves a number of important roles in the viral infection cycle. Its abundance suggests that it could be one of the essential elements for virion morphogenesis and assembly. It takes part in budding and aids in the assembly of all other structural proteins (S, E, and N) (Lopandić et al., 2021).

ELISA has four types, Direct ELISA, the sample is directly applied to the plate's surface. It is possible to measure an antibody that has been enzyme-tagged. After incubation, the unbound antibodies are removed from the media by washing, the appropriate substrate is added to the medium, resulting in a color signal that is proportional to the amount of antigen present in the sample. From a standard curve, this correlation can be used to know the concentration of antigen in an unknown sample. High molecular weight antigens can be determined by direct ELISA (Wang and D, 2019). Indirect ELISA , the antigen coated on wells, then primary antibody and a

labeled secondary antibody are used, which is a two-step binding process. The primary antibody is incubated with the antigen-coated wells in this approach. The secondary antibody is then added, which is tagged and recognizes the primary antibody. A polyclonal antibody is usually used as secondary antibody. Secondary antibodies that have been tagged are widely available. The well is subsequently filled with a substrate, which produce the signal. This approach is often used to identify bacterial, viral, or parasitic infection as well as measure antibodies against a foreign antigen (Schmidt et al., 2012).

Sandwich ELISA used to capture antigens in the well for detection, use two antibodies specific to the antigen. Antigen concentration and substrate response are directly related in immunometric tests. a capture antibody is usually coated on the plate. The antigen is bound by a second detection antibody that is also specific to the antigen during a second incubation. A secondary antibody-enzyme conjugate can bind the detection antibody, or the detection antibody can be enzyme-conjugated itself. When chromogenic substrate is introduced to the test to develop color, samples with higher antigen concentrations produce more signal than samples with lower antigen concentrations, yielding a signal that is proportionate to the amount of antigen in the sample (Kragstrup et al., 2013).

Competitive ELISA in which antigen in a sample competes with antigen attached to a reporter enzyme for restricted antibody binding sites. As a result, antigen concentration and substrate turnover have an inverse relationship. In competitive ELISAs, a single antibody to a low molecular weight antigen (less than 10,000 Daltons) is used. Unlabeled antigen is bound in larger levels than conjugated antigen during incubation in samples with high antigen concentration. When chromogenic substrate is added to the test to develop color, samples with a high antigen concentration provide a lower signal than samples with a low antigen concentration, resulting in an inverse relationship between antigen concentration in the sample and color development in the assay (Crowther and J. R, (2008).

In this study, indirect ELISA was chosen to test the antibody in the samples reacts with the coated antigen in the plate, as this type of ELISA considered to have high sensitivity and high specificity (Elshal et al., 2006).

2.8.3 Non-laboratory Diagnostic Method

Other frequently mentioned diagnostic techniques include surface plasmon resonance (SPR)-based biosensors, field-effect transistor (FET)-based biosensors, chest computed tomography (CT) scan combined with the assessment of clinical symptoms, and artificial intelligence techniques. CT is a regularly used supplementary detection method for the diagnosis of many illnesses. The SARS-CoV-2 infection's effects on lungs imaging can be used to make the diagnosis of COVID-19 (Caruso et al., 2020).

Because RT-PCR occasionally reported false-negative findings, it was discovered that CT scan was more sensitive for diagnosing COVID-19 than RT-PCR. CT scans are routinely used to better visualize lung abnormalities. The main drawback of the CT scan is its low specificity, which makes it impossible to distinguish between pulmonary abnormalities linked to etiological agents other than SARS-CoV-2 and the risk of ongoing exposure to radiation. The CT scan, in specific, has an excellent resolution power and a sensitivity of 95–100% (Sharma et al., 2021).

2.9 Treatment and vaccination

Nirmatrelvir/ritonavir (marketed as Paxlovid) or remdesivir can be used by patients with mild to moderate symptoms who are in the risk groups to lessen their likelihood of developing a serious illness or needing hospitalization. The Test to Treat project, part of the US Biden Administration's COVID-19 action plan, allows patients to visit a pharmacist, take a COVID test, and receive free Paxlovid right away if their results are positive. The core of COVID-19 management has been supportive care, which includes medicine or devices to assist other compromised essential organs, hydration therapy, oxygen support, and prone positioning if necessary (Wang et al., 2020).

Most COVID-19 instances are minor. Supportive therapy in these cases entails the use of medications like paracetamol or NSAIDs to treat symptoms (fever, body pains, cough), as well as adequate fluid intake, rest, and nasal breathing. A healthy diet and good personal cleanliness are also advised. The U.S. Centers for Disease Control and Prevention (CDC) advised anyone who believe they are infected to stay at home and wear a face mask as of April 2020 (U.S. Centers for Disease Control and Prevention, 2020).

In order to lower the risk of fatality in those severe cases, they were treated in hospitals with low oxygen levels, the use of the glucocorticoid dexamethasone was strongly advised. Support for

breathing may be needed through noninvasive ventilation and, ultimately, admission to an intensive care unit for mechanical ventilation. The advantages of extracorporeal membrane oxygenation (ECMO), which has been used to treat respiratory failure, are still being debated. Some cases of severe disease progression are brought on by cytokine storms (Kim et al., 2021).

Highly effective vaccines have reduced mortality related to SARS-CoV-2, which works by increasing immune response and preparing the immune system to recognize and kill the virus when it comes into contact with it. Live attenuated virus, inactivated virus, nucleic acid (DNA and RNA), protein subunits, and viral vectors have all been employed in the production of vaccines (Khade et al., 2021)

The Pfizer-BioNTech vaccine contains a small amount of harmless messenger RNA and is suggested for those aged 5 and older (mRNA). Body cells are given instructions by COVID-19 mRNA on how to establish an immunological defense against the virus that causes COVID-19. You are now less likely to contract COVID-19 in the future thanks to this reaction. After the immunological response has occurred, the body eliminates all vaccine components, just like it would any chemical that the cells no longer require. This is a typical process that the body goes through (Tregoning et al., 2020).

Long-lasting immunity is provided by the Sputnik Russian vaccine, which gets better 4-6 months after immunization. It is based on adenovirus DNA that carries the coronavirus gene for SARS-CoV-2. Adenovirus is used as a "container" to introduce a possible threat to the immune system by transferring the coronavirus gene to cells and starting the construction of the SARS-CoV-2 virus's envelope proteins. The cells use the gene to produce the spike protein after that. The immune system will identify this spike protein as being foreign and produce antibodies and T cells as natural defenses (Tregoning et al., 2020).

AstraZeneca vaccine: OXFORD vaccine uses chimpanzee adenovirus instead of human adenovirus and operates on the same basis as the sputnik. The vaccine adenovirus from the AstraZeneca injection attaches to and penetrates the cells of our own body, carrying the genetic code for the SARS-CoV-2 spike protein. Once within our cell, it moves to the nucleus to provide the genetic code. The altered vaccination adenovirus lacks the genes that a typical virus would use to replicate. The spike protein can only be produced by it. The genetic code of the COVID-19

spike protein is translated into messenger RNA (mRNA). With the assistance of the cell's protein-making machinery, this mRNA exits the nucleus and puts together copies of the COVID-19 spike proteins. Protein fragments are pushed outside of the cell and shown on the cell surface (Viner et al., 2020).

For those who are at least 18 years old, the Moderna vaccine against COVID-19 is recommended. It also includes a little amount of messenger RNA, which is safe (mRNA). Body cells receive instructions from COVID-19 mRNA on how to develop an immune response against the virus. You are shielded from getting COVID-19 in the future by this response. The body discards all vaccination components after an immune response has been produced, just like it would any chemical that the cells are no longer in need of. This is a typical aspect of how the body works (Viner et al., 2020).

Sinopharm Vaccine: The Chinese vaccine, which contains the fully intact and attenuated COVID19 virus, would be detected by the immune system and offer it the opportunity to begin building antibodies. According to Sinopharm, its effectiveness rate was 79 percent (Khade et al., 2021).

3. Materials and method

3.1. Samples Collection

Study location and population: this study was done in Al-Quds University Abu-Dis facilities - Palestine. the study was done on samples from different regions from Hebron and nearby villages. Two types of samples were collected:

1- Positive nasopharyngeal samples that were used for Covid-19 cDNA preparation, these samples were chosen randomly from Hebron Central Corona Lab, the prepared cDNA was used as a source for viral gene cloning. The collected cDNA samples were kept at -20^{o}C until their further use.

2- Serum sample of different Covid-19 conditions. These serum samples were mainly used for evaluating the use of the cloned Covid-19 antigens in ELISA test. A total of 56 serum samples that were used in this study: 13 samples of infected and vaccinated individuals, 25 serum samples of vaccinated individuals and their sera was collected 2-3 months post the second vaccination, 18 serum samples of different aged vaccinated individuals; their sera were collected at least 1.5 years after their second vaccination. Samples were collected between 27 August 2022 and 30 September 2022. The positive sera were chosen based on the development of COVID-19 symptoms and the presence of a positive COVID-19 RT-PCR and positive Rapid Ag test.

Study control group: Negative samples had been taken from Al-Quds University reserved frozen serum samples before pandemic starts, before 2019 the number was 4 samples for each 96 well plate. Positive control group is RT-PCR positive samples for SARSCoV2.

Ethical consideration: This study's proposal was submitted to Al Quds University-Medical laboratory science research committee for approval. All the participants were informed about the study aim and objectives and signed a consent form before participating as they had be notified about their rights to refuse, participate or withdraw at any time.

3.2. Cloning of SARS CoV-2 spike (S) protein and membrane (M) protein:

Generally, this was done by amplification of both S and M SARS CoV-2 protein gene segments by PCR, then ligation in pET-28a expression vector, and transformation into *Escherichia coli BL21* cells. The below steps indicate the specific detailed procedure for this cloning.

3.3. Amplification of SARS CoV-2 Surface and Membrane gene segments by Polymerase chain reaction:

A newly designed specific primers for S and M gene sequences were used for this purpose. Table 1 shows the exact sequence of the different used primers. These primers were designed based on previous cloning experiments in which the used primers introduced one extra nucleotide and hence creating frame shift mutation upon translation. In Table 1, the newly designed primers are shown in comparable with the old used primers. More details about primers the current primer design in results section.

Table 1: Sequences of the currently used primers. The yellow highlighted fonts indicate the added nucleotides after the chosen cutting site.

Gene Segment	New primers		Old primers
	Sequence	**Restriction enzyme**	**Sequence**
Membrane	**Direct:** GGG GGA GCT CCA TGG CAG ATT CCA ACG GTA CTA TTA C	SacI (GAGCT/C)	Direct: GGG GGA GCTC TGG CAG ATT CCA ACG GTA CTA TTA C
	Reverse: GGC GAA GCT TCT GTA CAA GCA AAG CAA TAT TGT CAC	HindIII (A/AGCTT)	**No changes**
Spike	**Direct:** GGG GGA ATT CAA TGT TTG TTT TTC TTG TTT TAT TGC CAC TAGT	EcoRI (G/AATTC)	GGGGCTCGAGATGTTTGTTTT TCTTGTTTTATTGCCACTAG
	Reverse: GGC GAA GCT TAG GGA GAT CAC GCA CTA AAT T	HindIII (A/AGCTT)	**No changes**

Each PCR reaction is composed of a total volume of 25 µl that contains: 12.5 µl of (2x ready mix Taq DNA polymerase mixture (Takara, Japan), 1 µl (20 pmoles) of direct and reverse primers for S and M primers separately, and 5 µl cDNA were added to each PCR reaction and double distilled water was used to fill the reaction volume up to 25 µl . The thermo-cycler program was

set as following: 5 min at 95°C; initial denaturation, 35 cycles each has 30 second at 95°C denaturation step, 30 sec at 53°C to perform annealing, followed by 1 min at 72°C as extension step (elongation) and a finally at 72°C for 10 min for final elongation.

3.4. Agarose gel electrophoresis

The PCR products were analyzed on agarose gel electrophoresis to ensure the sequence amplification PCR. 1.7 g of agarose was dissolved in 100 ml 1x TAE buffer (50X TAE running buffer contains (242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (pH 8.0)). Agarose was dissolved in TAE buffer and then 10 µl of Ethidium Bromide was added. after the gel was solidify, 7 µl of each PCR products were loaded to each well, and electric current was applied (120 voltage for 30 minutes). 100 bp ladder (Thermo Scientific) was used as a size marker. Results were documented by gel-documentation system.

3.5. Restriction enzyme cutting for Surface (S) and Membrane protein (M) insert gen.

The amplified S and M genes were precipitated by standard ethanol precipitation method. About 1 µg from each gene was cut by two restriction enzymes (SacI and HindIII) for M gene amplified segment, and (EcoRI and HindIII) for S gene amplified segment. Restriction cutting was done according to the standard cutting protocols and according to the manufacturer instructions (NEB, USA). Simply restriction cut was done in 50 µl reaction with at least 100 units of restriction enzyme. The same used restriction cutting enzymes were used to digest the used cloning plasmid (pET-28a). The digested DNA was precipitated by standard ethanol precipitation protocol to remove salts and to concentrate the DNA in small volume suitable for ligation reaction.

3.6. Ligation and transformation of surface and membrane gene insert in pET-28a vector.

Ligation reactions between spiked and membrane DNA gene segments from one side and the digested plasmid were performed in a reaction volume of 20 µl at 16 °C for 24 hours. The ligation reaction included: 10 µl of the pET-28a expression vector ,7 µl of surface or membrane gene, 1 µl

of T4 DNA ligase enzyme (Thermo-Scientific, USA) with its buffer in a total volume of 20 µl. Later the ligation reactions for each S and M gene segments were transformed to *E. coli BL21* competent cells by heat chock transformation method. The transformed bacteria cultured on LB agar plates with 50 µg/mL kanamycin antibiotic.

3.7. S and M clones isolation.

The transformed bacterial Bl21 E. coli cells were picked and cultured on LB agar by streaking method and incubated for overnight at 37°C, then single colony was taken and cultured in 5 ml LB broth and incubated at 37°C with shaker overnight. 500 µl of the bacterial cells were lysed by boiling for 10 minutes to release its genome, then the free genetic material was obtained by centrifugation for 5 minutes at high speed (14,000 rpm) followed by PCR using standard T3 and T7 primers found on the used plasmid vector.

3.8. Purification and Sequencing of M and S amplified DNA segments.

PCR products were purified by PCR purification Kit (Qiagen, Germany), and according to manufacturer's instructions. The amplified PCR DNA products were added into eppendorf tube and their volume was increased by the addition of 100µl of sterile double distilled water. This was followed by the addition of 300µl of binding buffer and the mixture was added into PCR purification columns followed by centrifugation for 60 sec to discard flow-through. 700µl wash buffer (ethanol) was added to the PCR purification column. The purified bound DNA was eluted by the addition of 20µl double distilled water to concentrate the purified DNA. The purified DNA was sent for DNA sequence analysis. Sequence analysis was done by dye terminator method, using Automated DNA Sequencer machine (AB477).

3.9. SARS CoV-2 Surface and Membrane proteins expression and purification.

Expression and purification of both S and M proteins from the produced and confirmed clones was done separately for each recombinant protein. Simply; this was done in 500 ml sterile flask, containing 30 ml of LB broth media, to it 30 µl of 50 µg/mL kanamycin antibiotic and 0.5 ml of the transformed overnight cultured bacteria was added. After 2 hours incubation at 37 ^{o}C , 30 µl of 1 M Isopropyl-ß-D-thiogalactoside (IPTG) was added then incubated overnight at 37°C with shaking in water bath. After 24 hours, the flasks were transferred to conical 15 ml tubes and centrifuged at 5000 rpm for 10 minutes, the pellet was taken and stored with PBS buffer at- 20°C. The produced pellet mixture in PBS was lysed using 0.5 ml of lysis buffer containing 10mg

lysozyme. The mixture was incubated at 60°C for two hours, followed by sonication. The whole lysed mixture was then centrifuged at high speed (14,000 rpm) for 10 minutes. The supernatant was collected and protein concentration was measured by reading the mixture absorbance with spectrophotometer at 280nm.

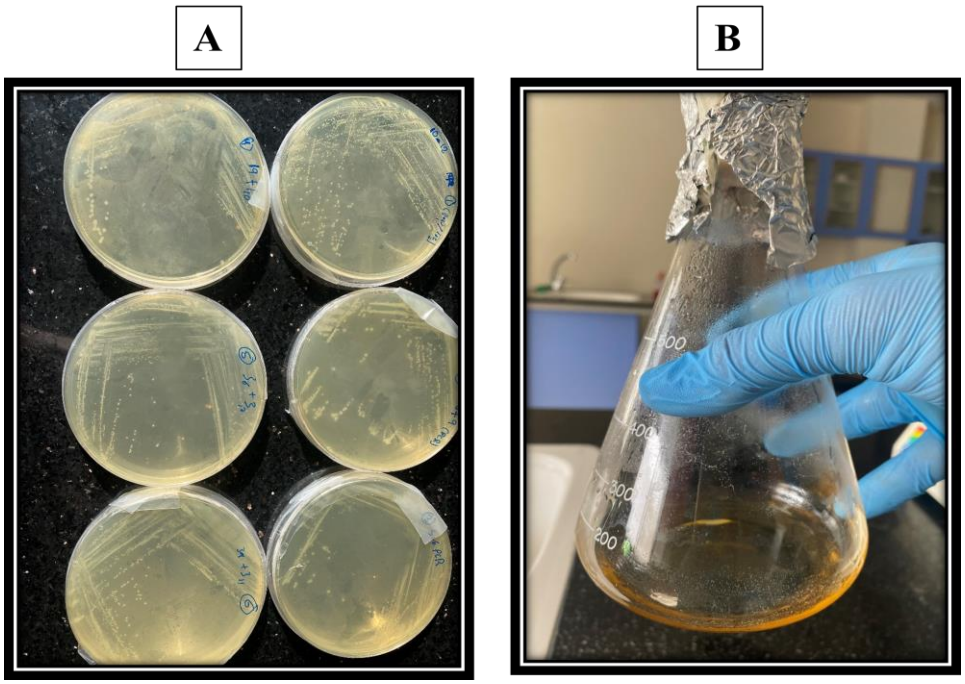

Figure 7: Isolation of single colony of S clone and M clones and their protein induction in 30 ml LB broth. A: streaking results showing single colonies, B: protein expression induction

3.10. Enzyme Linked Immunosorbent Assay (ELISA) preparation using the new recombinant antigens.

The extracted crude protein concentration was 0.5 mg/ml, its diluted in 1x PBS to 25µg /ml. (10 x PBS contain 2.4 g KH_2PO_4, 17.8 g Na_2HP_4, 80 g NaCl). The first step in ELISA is coating the recombinant antigen (Surface protein and Membrane protein) into two separate 96 wells plates ,100 µl of the recombinant was coated overnight at 4C. Blocking step done by adding 200 µl of blocking buffer (PBS, 5% FCS: Fetal Calf Serum- sigma-Aldrich, Sant-louis-USA, 0.05%Tween-20) for 20 minutes at room temperature then the blocking buffer was removed without washing. Then, the samples and the negative and positive controls was serially diluted in the blocking buffer as follow (1:20, 1:40, 1:80) ,100 µl of the diluted sample was added as duplicate and incubated at

room temperature for two hours. the blank of every plate was the blocking buffer. After that, 3 times washing step was done by adding 200 µl of washing buffer (PBST: 1x PBS, .05% tween20). The step after that is adding the secondary antibody protein-A conjugated to HRP to detect IgG antibody in the tested samples, the secondary antibody diluted to 1:5000 and 100 µl was added to each well, incubated at RT for one hour, followed with 3 time washing as mentioned. Finally, addition of 200 µl of substrate, the substrate mixture was done as follow: (2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) ,20 ml of 0.1M tri-sodium citrate buffer (pH5) and 20µl of H_2O_2) and incubated a RT for 30 minutes. after that the absorbance was measured by ELISA reader at 405 nm after blanking each plate.

4.Results

4.1. Discovery of point mutation in previously cloned S and M gene segments.

In a previous work our lab was able to clone both S and M gene segments to be used in their expression. The cloning protocol used restriction cut sequences (SacI, HindIII) that is linked at the 5' end of the designed primers. So, and upon digestion of the amplified PCR products of both S and M DNA gene segments; new nucleotide sequence was introduced at the 5'end of both S and M genes. These inserted nucleotides created frame shift mutations that produced different amino acid sequence compared to the original S and M amino acid sequences. In this current study new nucleotides were introduced in the currently used direct primers for both S and M. The introduced nucleotides were not related to S and M DNA gene segments, but they enabled to keep the original expressed amino acid sequence of these protein. As it could be seen in Table 1, two nucleotides were inserted after SacI cut site in the direct primers used for M gene amplification, and one nucleotide was inserted after EcoRI restriction site in direct primer used in S gene amplification.

4.2. Amplification of S and M DNA gene segments:

10 PCR positive samples that collected from Hebron central corona lab, Palestine used to amplify SARS Cov2 surface gen and SARS Cov2 membrane gens to be used later for cloning in pET-28a plasmid. Figure 7 depicted the results of this amplification by agarose gel electrophoresis analysis. The amplified DNA products using M direct and reverse primers were composed of single unique band with an approximate size of 700 bp, while 2-3 banding pattern was obtained using S primers. The amplified bands from the 10 positive samples either of S or M DNA gene segments were grouped together (One tube for M and another for S gene segments). This PCR products was used as a rich source for restriction enzyme digestion and later cloning in pET28-a plasmid.

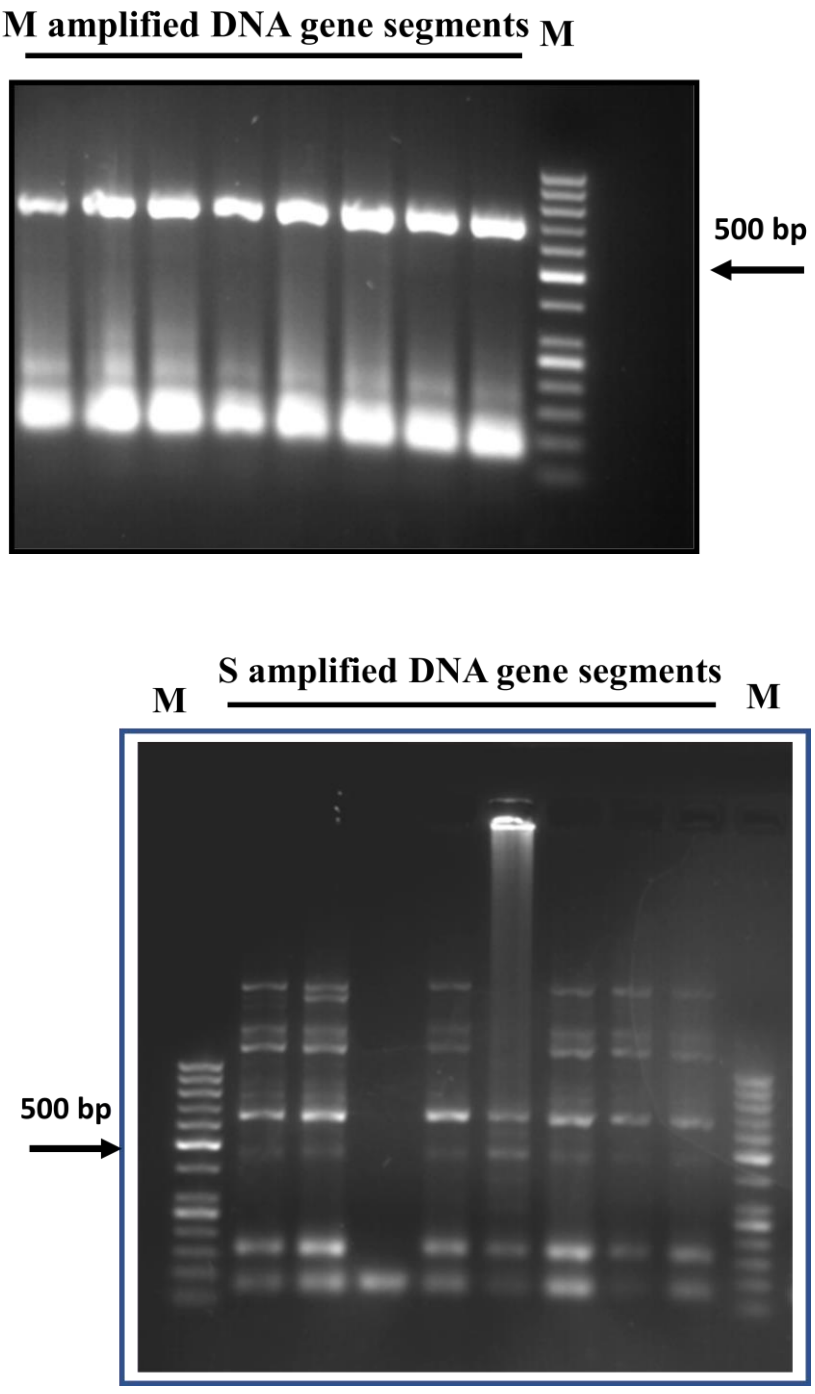

Figure 8: Agarose gel electrophoresis analysis shows the amplified PCR DNA gene segments targeting Membrane protein (upper) and Surface protein gene (lower) from SARS CoV-2 positive samples. M: 100 bp DNA size marker.

Figure 9 shows the result of gel electrophoresis for PCR product of the extracted genome from the clones that was suspected to have the gene of interest, the M gene is giving a clear single band which suggest its suitability for the selected way of sequencing.

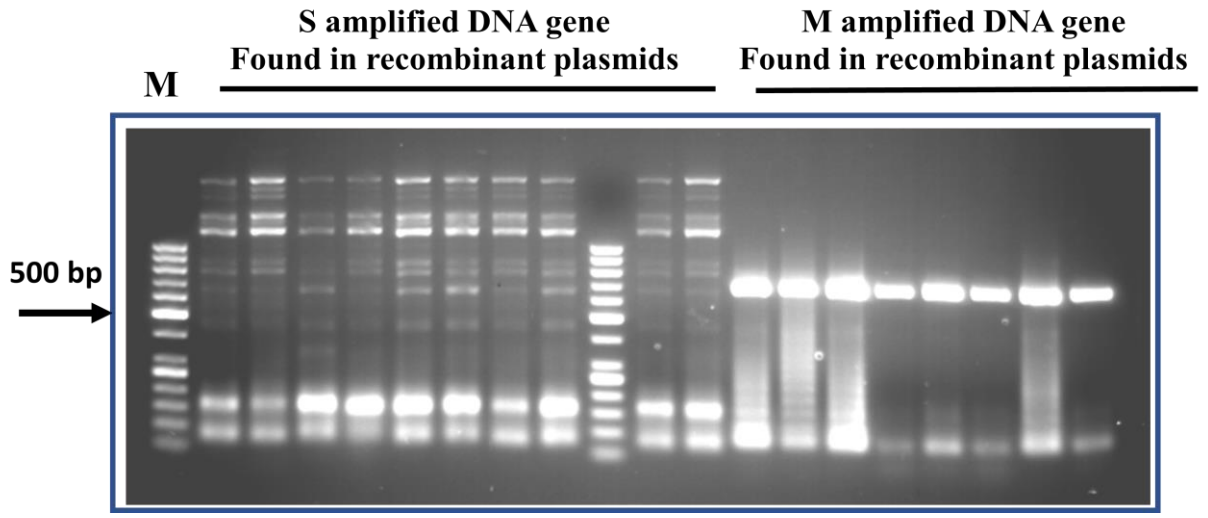

Figure 9: Agarose gel electrophoresis analysis showing the PCR products that was amplified from recombinant plasmids containing Cov-2 DNA insert segments of both surface and membrane genes. M: 100 bp DNA size marker.

4.3. DNA sequence analysis of cloned recombinant surface and membrane DNA genes.

In part of this study, it was only possible to do DNA sequencing for M DNA cloned gene. The fact that the amplified PCR products using S primers produced more that strong band, this imposes restriction on the use of Sanger DNA sequence analysis. In the future it is possible to confirm the S DNA gene segment by the use of next generation sequence analysis for the whole recombinant plasmid.

Regarding the cloned M DNA gene, we confirmed its presence by direct sequence analysis from the amplified PCR products. For this purpose, we sent 4 samples for DNA sequencing and we used reverse and direct primers for sequence analysis.

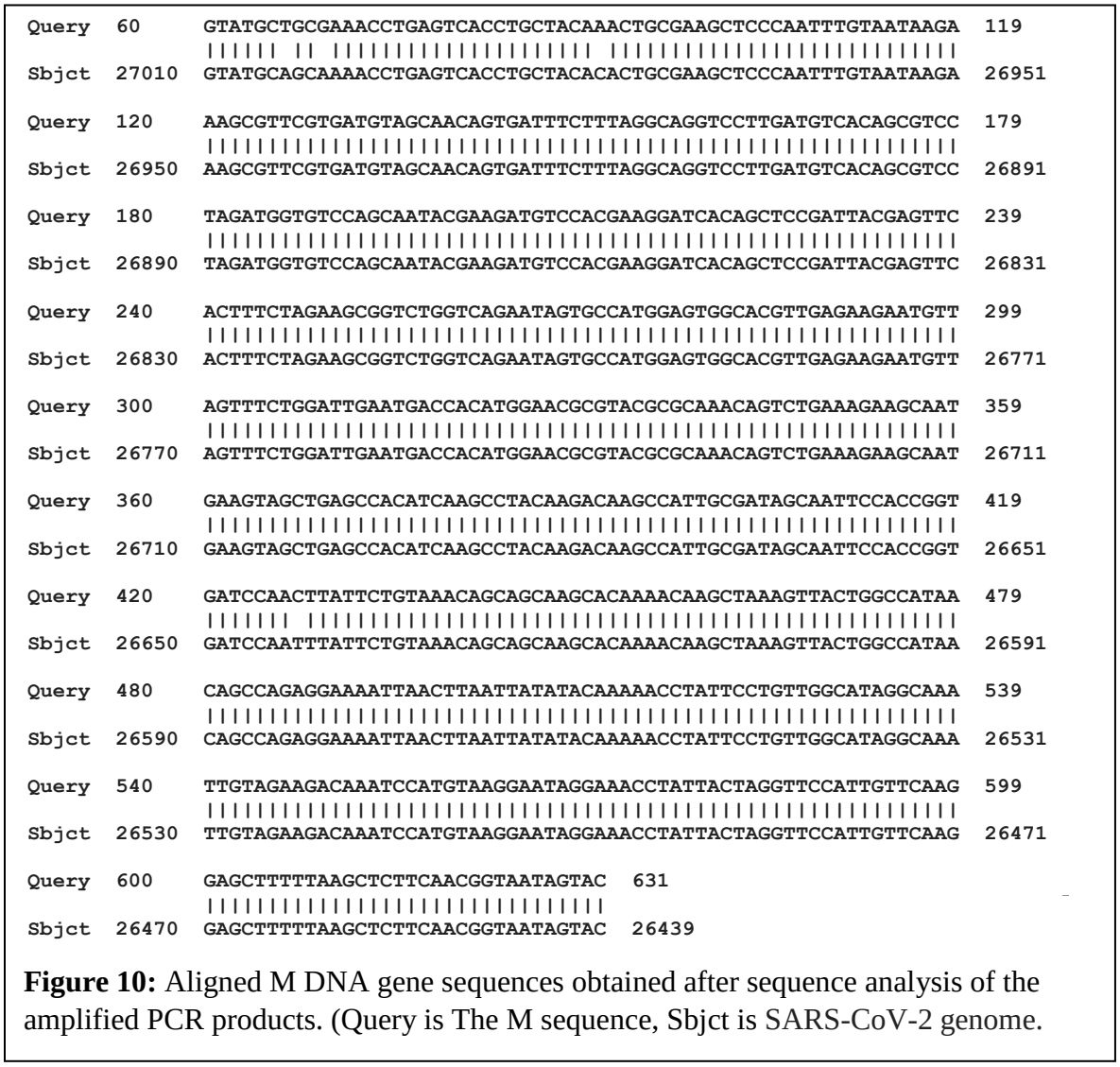

Figure 10: Aligned M DNA gene sequences obtained after sequence analysis of the amplified PCR products. (Query is The M sequence, Sbjct is SARS-CoV-2 genome.

BLAST analysis of the obtained sequencing data for M DNA gene segments showed a similarity of (99.3%) with sequences of SARS-CoV-2 genomes found in GenBank (Figure 09). The aligned sequence covering a region between 26439 bp up to 27010 bp in SARS-CoV-2 genome, this site is responsible for coding the viral membrane (M) protein.

4.4. S and M proteins expression and purification.

After IPTG was added to induce the bacteria clone to express the Cov-2 surface cloned protein and the Cov-2 Membrane cloned protein. Then the recombinant protein was extracted, and its concentration was measured by spectrophotometer to be 0.5 mg/ml in order to be diluted to 25µg/ml and to be used in coating ELISA plates.

4.5. Using the recombinant Cov-2 surface and membrane proteins in screening of serum samples by Enzyme Linked Immunosorbent Assay (ELISA):

The recombinant S and M proteins were used in and ELISA serological tests to evaluate their use in future screening purpose. Both extracted crude proteins were used in a diluted of 25 □g/ml in PBS. 96 microtiter ELISA plates were coated with these recombinant proteins (each in different plate). A total of 56 samples were used that were arranged into three groups: **1-** 13 samples of infected and vaccinated individuals. **2-** 25 serum samples of vaccinated individuals and their sera was collected 2-3 months post the second vaccination. **3-** 18 serum samples of different aged vaccinated individuals; their sera were collected at least 1.5 years after their second vaccination. All serum samples were tested against M and S proteins after doing the following dilutions: (1:20, 1:40, 1:80). Blocking buffer is used as blank in every plate and stored serum before pandemic was used as negative controls in every plate as well.

Tables 2-4 show the antibody titers obtained by ELISA test in comparative manner upon coating with S or M recombinant proteins. In general, the total number of positive samples using S proteins in ELISA test was relatively higher compared to obtained positive samples using M recombinant proteins, the numbers were 39 positives using S proteins and 36 positives using M protein. More important it was the antibody titers that are obtained in the positive's samples using S protein as an antigen were much higher than those antibody titers that were obtained using M proteins.

It was clearly seen that serum samples that were previously infected and then vaccinated have a higher rate of positivity (100%) using any of the two antigens in ELISA test, and these samples showed the highest measured antibody titers (Table 2). Table 3 showed the results of antibody titers of tested serum samples that were collected 2-3 months after vaccination, there is a higher percentage of positivity and higher antibody titers compared to serum samples collected 1.5 years after vaccination (Table 4). In general S recombinant protein proved more suitable for evaluating antibody titers in SARS-CoV-2 vaccinated groups, and it is clearly seen that antibodies titers are decreased and the time relapse after vaccination.

Table 2: Antibody titers of infected and vaccinated serum samples tested against S and M protein antigens in an ELISA test.

Sample Number	Antibody titer S Antigen	Antibody titer M Antigen
1	1:40	1:40
2	1:80	1:80
3	1:80	1:40
4	1:80	1:80
5	1:80	1:40
6	1:80	1:80
7	1:80	1:40
8	1:80	1:40
9	1:80	1:20
10	1:80	1:20
11	1:80	1:20
12	1:80	1:40
13	1:80	1:20
Total positive samples	**13**	**13**

Table 3: Antibody titers of vaccinated (2-3 months after vaccination) serum samples tested against S and M protein antigens in an ELISA test.

Sample Number	Antibody titer S Antigen	Antibody titer M Antigen
1	1:20	1:20
2	1:20	-ve
3	1:40	1:80
4	-ve	-ve
5	1:20	1:20
6	1:20	1:20
7	1:20	1:20
8	1:20	1:20
9	1:40	1:40
10	1:20	-ve
11	-ve	1:20
12	1:20	1:40
13	1:20	-ve
14	1:20	1:20
15	1:80	1:40
16	-ve	-ve
17	1:40	1:20
18	1:20	1:20
19	1:20	-ve
20	-ve	-ve
21	1:20	-ve
22	1:20	1:20
23	-ve	-ve
24	-ve	-ve
25	1:20	-ve
Total positive samples	**19**	**14**

Table 4: Antibody titers of vaccinated (at least 1.5 years after vaccination) serum samples tested against S and M protein antigens in an ELISA test.

Sample Number	Antibody titer S Antigen	Antibody titer M Antigen
1	1:20	-ve
2	-ve	-ve
3	1:20	1:20
4	1:40	1:20
5	-ve	-ve
6	1:20	-ve
7	1:20	-ve
8	-ve	1:40
9	-ve	1:20
10	-ve	1:20
11	1:20	1:40
12	-ve	1:20
13	-ve	1:20
14	-ve	1:20
15	-ve	-ve
16	-ve	-ve
17	-ve	-ve
18	1:20	-ve
Total positive samples	**7**	**9**

5. Discussion

SARS CoV-2 was first identified in Wuhan as a serious a respiratory disease. The disease was conserved as a pandemic affecting many countries with symptoms that include fever, cough, difficulty breathing. The virus is primarily spread through respiratory droplets. There is currently no specific treatment for this disease, but research is ongoing to develop vaccines and treatments. Serological monitoring of SARS-CoV-2 vaccinated individuals has become an essential component in understanding vaccine effectiveness and assessing immune responses. Serological tests measure the presence and levels of antibodies generated by the immune system after vaccination. These tests provide valuable insights into the immune status of vaccinated individuals and help evaluate the duration and strength of their immune protection against the virus.

One key objective of serological monitoring is to determine the vaccine's ability to elicit a robust antibody response. By measuring the levels of specific antibodies, such as anti-Spike protein antibodies, scientists can assess the vaccine's efficacy in stimulating an immune response. High antibody titers suggest a strong and effective immune reaction, indicating a higher likelihood of protection against SARS-CoV-2 infection. Monitoring antibody levels over time can also help identify any decline in antibody titers and guide decisions regarding booster doses or the need for additional vaccinations. Furthermore, serological monitoring can aid in assessing the duration of vaccine-induced immunity. By regularly testing vaccinated individuals, researchers can track the persistence of antibodies over months or years. This information is crucial in understanding the longevity of vaccine protection and determining the optimal timing for potential booster shots. Additionally, monitoring antibody levels can provide insights into the waning of immune response, especially among specific populations such as the elderly or immunocompromised individuals who may exhibit different antibody kinetics.

Recombinant antigens are playing a pivotal role in serological tests for SARS-CoV-2. These tests detect the presence of antibodies in an individual's blood, aiding in the identification of past infections or immune responses to the virus. Recombinant antigens are synthetic proteins that mimic specific parts of the SARS-CoV-2 virus, allowing for accurate and reliable testing.

One of the main advantages of using recombinant antigens is their high specificity. By selecting and producing specific viral proteins, such as the spike (S) protein or nucleocapsid (N) protein. Using these antigens ensures that the serological test can accurately differentiate between antibodies generated in response to SARS-CoV-2 and those generated due to other coronaviruses or unrelated pathogens, reducing the chances of false-positive results.

Recombinant antigens also offer scalability and reproducibility. These proteins can be produced in large quantities using biotechnological methods, ensuring a stable and consistent supply for widespread testing. Unlike traditional methods that rely on the cultivation of live viruses or viral fragments, recombinant antigens provide a safer and more controlled approach. They eliminate the risks associated with handling infectious materials and allow for standardized production, resulting in uniform test performance across different laboratories. Furthermore, recombinant antigens can be engineered to enhance their detection properties. By incorporating specific tags or markers, such as fluorescent or enzyme labels, they enable easy visualization or quantification of antibody-antigen interactions. This simplifies the interpretation of test results and facilitates high-throughput screening, making them suitable for large-scale serosurveillance studies or population-level assessments.

This study has successfully produced recombinant SARS CoV-2 Surface and Membrane Protein, and used them to develop immunological test for detection of SARS CoV-2 IgG antibodies in qualitative and quantitative manner, this method will provide continuous unexpensive source of antigenic material for improving immunological tests like our developed ELISA test.

Escherichia coli strain used to clone, sequence, and express the SARS-CoV1 nucleocapsid (N) protein. Using an ELISA test, the SARS-CoV1 specific IgG antibodies from the serum of 16 SARS-CoV1-infected patients and 131 control persons were measured using purified N protein. After 10, 20, and 30 days since the start of the disease, specific antibody responses to the purified recombinant N protein were detected in the sera of 13 of 16 (81.3%), 16 of 16 (100%), and 16 of 16 (100%) SARS patients, respectively (Timani et al., 2004).

Serological tests that available for measuring SARS-CoV-2 antibodies use different antigens, their specificity and sensitivity still a challenge. Our study aimed to develop an "in-house" serological ELISA to measure SARS-CoV-2 antibodies concentrations by using the two different protein antigens (SARS CoV-2 Surface and Membrane protein) that recombinantly cloned in Al-Quds University labs.

The sensitivity and specificity of the commercially available SARS-CoV-2 serological tests, which are used to identify antibodies against S and N protein antigens, vary due to changes in the time of performance and study population. The most popular antibodies for detecting SARS-COV-2 infection in commercial ELISAs, which are often delivered as pre-coated plates, are IgM and IgG or mixed IgG and IgM antibodies (Vengesai et al., 2021).

According to a meta-analysis done recently, most studies' sensitivities and specificities, respectively, ranged from 80% to 100% and 95% to 100%. Zhao in 2020 assessed the sensitivity for all ELISA tests based on IgG (n = 10) about 0.65 (95% CI 0.57-0.72). Kai-Wang's estimate was 1.00 (95% CI 0.79-1.00). Ling Zhong in 2020 estimates specificity from 0.86 (95% CI 0.51-0.89) to 1.00 (95% CI 0.98-1.00) (Vengesai et al., 2021).

So, for all the ELISA tests based on IgG, the sensitivity estimates ranging from 65% to 100%, and specificity estimates ranging from 86% to 100% (Lagousi et al., 2021). Thus, our developed ELISA using our cloned SARS-CoV-2 antigens (S and M) as capture antigens had comparable specificity and sensitivity than commercially available ELISA diagnostic kits. Our "in-house" ELISA corresponds with other commercial assays that report high sensitivity for IgG antibodies.

The diagnostic evaluation of specific antibodies against the SARS-CoV-2 virus is mainly depending on spike (S) and nucleocapsid (N) proteins; however, the most abundant membrane (M) protein is largely ignored in SARS-CoV-2 serology testing (Lopandić et al., 2021).

Other viral proteins, however, trigger an immunological response. There is currently little data on the usage of M protein in COVID-19 serology assays. As a result, this study in addition to S protein, also investigated the immunoreactivity of recombinant M protein with IgG antibody in

serum samples from COVID-19 infected, convalescent, and vaccinated people. Our recombinant developed and purified M protein revealed good IgG reactivity with serum samples from COVID-19 infected, convalescent, and vaccinated people.

According to a 2021 study, The M protein's outer N-terminal section (19 aa) and internal C-terminal tail (101-222 aa) were designed in silico and recombinantly synthesized and purified. Western blot and ELISA were used to assess the developed M protein. The M antigen was tested by western blot and demonstrated IgG and IgM reactivity with serum samples from COVID-19 convalescents. The M antigen was also tested in ELISA, more than 93% (28/30) of COVID-19 sera tested positive for IgM and 96% (29/30) tested positive for specific IgG to M protein. (Lopandić et al., 2021).

Regarding S protein, a study in April 2020, have optimized an ELISA against SARS-CoV-2 spike protein, the study used diluting sera 1:100 and goat anti-human Pan Ig secondary antibody which maximized sensitivity and specificity of the assay. With specificity more than 99%, and a sensitivity of 96%, the study concluded that ELISA assay can be used to identify prior SARS-CoV-2 infections without molecular diagnostic confirmation (Freeman et al., 2020).

This assay can be adjusted for use in higher throughput serosurveys and in combination with confirmatory testing could be used in an algorithm for informing individuals of prior infection

In 2021, Lopandić used 20 sera samples , half of them positive with SARS-CoV-2 and the other half is negative, these samples were used to test SARS-CoV-2 IgG and IgM reactivity by ELISA assay, to optimize the new assay based on SARS-CoV-2 M protein. When utilizing M protein as the coated antigen at a final concentration of 2 g/mL, the largest variation in OD values for negative and positive sera for both classes of antibodies was observed (OD 0.054–1.750 for IgG, OD 0.067–1.547 for IgM) (Lopandić et al., 2021).

Lopandić also concluded that the tested sera had an optimal dilution of 1:50. The optimal dilution of HRP-conjugated anti-human IgG and anti-human IgM was discovered to be 1:5000 and 1:8000, respectively, using this coated protein and serum concentration. The best cut-off value for

IgG detection was 0.220 OD, based on that, sensitivity and specificity was 96.7% and 92.5% respectively (Lopandić et al., 2021).

Serological monitoring of SARS-CoV-2 vaccinated individuals is instrumental in evaluating vaccine efficacy, understanding immune responses, and guiding public health measures. By assessing antibody levels, duration of immunity, breakthrough infections, and population-level effectiveness, serological monitoring helps optimize vaccination strategies, identify at-risk populations, and inform policies to control the spread of COVID-19. Continued monitoring and research in this field will contribute to our understanding of long-term vaccine protection and aid in the ongoing fight against the pandemic.

In conclusion, the use of recombinant antigens in serological tests for SARS-CoV-2 has revolutionized the field of diagnostics. Their high specificity, scalability, reproducibility, and flexibility make them an indispensable tool for accurate and efficient detection of antibodies against the virus. As the global fight against COVID-19, continues recombinant antigens will continue to play a crucial role in serological testing, enabling us to better understand the spread of the disease, monitor immune responses, and inform public health measures.

And finally, I would recommend to test the developed antigens on large numbers of samples from different areas in Palestine, to assess the sensitivity and specificity of the developed ELISA, to test the developed antigens on each given vaccine, to assess every vaccine effectivity, and to purify the extracted antigens using any purification technique like nickel affinity chromatography to have purer product.

الملخص باللغة العربية :

العنوان: تطوير (ELISA) اختبار التشخيص المناعي للكشف عن المصابين ب SARS-CoV-2 والمطعمين ضده من خلال فحص الامصال البشرية

اعداد : روند ناجي طلب عبد الجواد العجلوني

اشراف : د ابراهيم عباسي و د رسمي ابو الحلو

المقدمة : SARS-CoV-2 (فيروس كورونا 2 المسبب للمتلازمة التنفسية الحادة الوخيمة) هو فيروس تاجي جديد ظهر مؤخرا قد تسبب في جائحة بشرية. على الرغم من التطور السريع لطرق التشخيص الجزيئي ، فإن التشخيص المناعي من خلال الامصال ضرورية للكشف ودراسة الاستجابة للتطعيم والبحوث الوبائية. كان الهدف الأساسي لدراستنا هو استنساخ بروتين SARS-CoV-2 السطحي (surface) والغشائي(membrane) واستخدامها لإنشاء ELISA غير مباشرة حتى نتمكن من فحص نجاعة البروتينات المستنسخة في امكانية استخدامها كانتيجينات في اختبار ELISA.

الطريقة : تم استنساخ البروتين الغشائي (M) وبروتين سبايك (S) من SARS-CoV-2 باستخدام بكتيريا *Bl21 E. coli* ، ثم تم إجراء اختبار ELISA خطوة بخطوة وتعديله في المختبر لاختبار العينات التي تم جمعها من المرضى المصابين والملقحين وغير الملقحين باستخدام مستضدات البروتين المستنسخة في ELISA

النتائج: أسفرت دراستنا عن استنساخ جين بروتين SARS-CoV-2 السطحي والغشائي في بكتيريا *Bl21 E. coli* بعد اكتشاف طفرة نقطية. حقق الجين المستنسخ حديثا تشابها بنسبة (99.3٪) مع تسلسل جينومات SARS-CoV-2 الموجودة في GenBank. تم استخلاص البروتين الخام بتركيز 0.5 مجم / مل. تم اختبار استخدام بروتينات S و M المستخلصة في أغراض الفحص المستقبلية باستخدام اختبار ELISA ، عينات المصل التي تم جمعها بعد 2-3 أشهر من التطعيم لها نتائج إيجابية أعلى (ن = 25/19) عند استخدام مستضد S مقارنة بمستضد M (ن = 25/14). كان عدد النتائج الإيجابية في عينات المصل التي تم جمعها بعد 1.5 سنة على الأقل من التطعيم 18/7 لمستضد S و 18/9 لمستضد M. كان إجمالي العينات التي تعطي نتيجة ELISA إيجابية 39 في مستضد S والعينات الإيجابية في مستضد M كانت 36 ، وكان titer الأجسام المضادة المرتبط بمستضد S أعلى من مستضد M بشكل عام . كان عيار الأجسام المضادة أعلى في العينات التي أصيبت سابقا ثم تم تطعيمها ، ثم العينات التي تم جمعها بعد 2-3 أشهر من التطعيم ، ثم أخيرا ، العينات التي تم جمعها بعد 1.5 سنة من التطعيم.

الخلاصة : تم استنساخ مستضدات S و M المؤتلفة بنجاح ، نوصي باستخدام مستضد S في اختبار ELISA بدلا من البروتين M ، أو استخدامها معا. نوصي أيضا بإجراء المزيد من الدراسات للحساسية والنوعية ، أو الدراسة الوبائية وزيادة عدد العينات في الدراسة ومن عدة مدن مختلفه في فلسطين .

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