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"Evaluation of antibody titers and serotypes in COVID-19 vaccinated human being"

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“Evaluation of antibody titers and serotypes in COVID-19 vaccinated human being”

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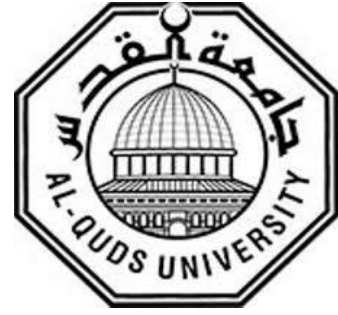

Thesis Approval

Evaluation of antibody titers and serotypes in COVID-19 vaccinated human being

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Dedication

I dedicate this thesis to my family and many friends who have meant and continue to mean so much to me.

I also dedicate this thesis to my teachers, and I appreciate every word have been said to me by my teachers.

Declaration

I hereby certify that this thesis is the result of my own research, except where the otherwise acknowledged, and that this study has not been submitted for a higher degree or institution.

Signed:

Ahmad Ziyad Abdelkader

Date: 04/01/2025

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Thank God the Merciful and Graceful.

I would like to express my profound gratitude to my research supervisor, Dr. Rasmi Abu Helu.

Special thanks to my family, friends, who encourage me constantly.

Abstract

Introduction: SARS-CoV-2 is a novel coronavirus that emerged in 2019 and is now classified in the genus *Coronavirus* with closely related SARS-CoV. Due to the high pathogenicity of this virus in humans, it is classified as biosafety level 3 (BSL-3) pathogen, which makes manipulating it relatively difficult due to its infectious nature that killed high number of people.

Methods: To assessment immune system and antibodies titer the serum samples collected from 85 hospitalized vaccinated patients against COVID-19 disease or were previously infected patients and from normal non vaccinated negative donors. To evaluation of antibody titers an Enzyme-linked immunosorbent assay (ELISA) technology performed in laboratories, and evaluation the virus infection, and proteins M and S spike proteins of SARS-CoV-2 evaluated and determined for detection of spike-binding neutralizing antibody titers in sera from hospitalized COVID-19 vaccinated patients by using commercial enzyme and ELISA technology. Furthermore, to measure SARS-CoV-2 neutralizing antibodies under BSL conditions, an ELISA for SARS-CoV-2 was developed. Recording the results of laboratory analyzes and saving them in a database using a computer.

Result: An analysis and assessment the antibodies titer was conducted and obtain results about the Corona virus, its spread and its effect on people, where the immunological and microbiological results of the people were analyzed and then enter this data as input into the laboratory database, and it was found from this study that there is a clear discrepancy between the data of vaccinated and healthy people, as it appeared that the vaccinated show higher titer than unvaccinated normal individuals.

In conclusion, this study aimed to evaluate the long-term effectiveness of COVID-19 vaccination in Palestine people by analyzing they response to the vaccine after two years, based on anti-SARS-CoV-2 protein S and M antibody titer levels. This study demonstrates the importance of knowing the impact of the Corona virus on people, efficacy of vaccines and presence of neutralizing antibodies against Covid19. To evaluate an immune system throw examination by assessment antibody titer and

serotypes specially using high specificity and sensitivity ELISA technology to COVID-19 system, which easily can be implemented and performed within a biosafety level laboratory (BSL) for evaluating the occurrence of neutralizing antibodies against SARS-CoV-2 and assessment of both S and M antigens to control and limit the transmission of the COVID 19 in order to find an appropriate treatment for this virus and improve new vaccination in Palestine.

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Abbreviations

Abbreviation	Abbreviation Representation
SARS CoV2	Sever Acute Respiratory Syndrome Coronavirus 2
ELISA	Enzyme-Linked-Immunosorbent Assay
E. coli	Escherichia Coli
IPTG	Isopropyl-beta-D-thiogalactoside
PBS	Phosphate Buffer Saline
HRP	Horseradish peroxidase
SDS-PAGE	Sodium-Dodecylsulfate Polyacrylamide Gel Electrophoresis
COVID-19	Corona virus disease 2019
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
cDNA	Complementary DNA
RT-PCR	Real Time Polymerase Chain Reaction
+ssRNA	Positive single stranded RNA
ACE2	Angiotensin-Converting Enzyme 2
D.D.W	Double distilled water

Chapter One

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that causes Coronavirus Disease 2019 (COVID-19), is very important illness which infect the respiratory systems .(Hui, 2020).

The COVID19 epidemic is regarded as the century's greatest significant worldwide medical disaster. The COVID-19 virus may spread rapidly making it simpler to replicate around the world (Indranil Chakraborty 2020 Apr).

It was originally discovered on December 31, 2019, in Wuhan, China, where it quickly turned into an emergency outbreak.(Hengbo Zhu, 02 March 2020)

More than 275 million instances and more than 5 million fatalities were reported globally on December 2020 according to the World Health Organization's official daily COVID-19 Situation Reports.(https://data.who.int/; Morin et al., 2008)

COVID-19 are positive-single-abandoned RNA (+ssRNA) infections with a crown like appearance spike glycoproteins projections on a superficial level.(Cascella, 2020).

During COVID19 outbreak between 2019 to 2020, the disease diagnosis has significantly impacted clinical microbiology laboratories, they were have to tackle problems such as fast and accurate diagnosis that was based basically on molecular diagnosis, the pre analytical step and/or collecting the right respiratory tract samples at the right time through the right anatomic place were challenging and crucial (Wang, 2004).

Compared to molecular assays, such as RT-PCR, serological assays for SARS-CoV-2 diagnosis are frequently less expensive, and a varieties of quick immunochromatography tests were available, that were testing IgG or IgM antibodies against COVID-19, these tests were aslo simpler for employees with no extensive laboratory training to do. The sensitivity and accuracy of detection can be improved by using the detection of COVID-19 antibody titer, either alone or in combination with PCR. Many commercial companies developed different serological assay kits in order to detect SARS- COVID-19 IgG, IgA and IgM antibodies have been certified upon FDA(Younes, 2020).
The best-known popular serological test for COVID-19 diagnosis is the enzyme-linked immunosorbent assay (ELISA), which is a sensitive methods to detect the antigen or antibody of interest within the serum samples. Enzyme-linked immunosorbent assays (ELISAs) are sensitive and specific, however they are dependent on the viral protein employed as a capture antigen.

COVID-19 antigen can be used in two ways: directly, where an enzyme-linked antibody binds to the antigen in the sample, or indirectly, where a primary antibody binds to an antigen that has been coated to the wells of a microtiter plate, and a secondary enzyme-labeled antibody is then applied to detect the primary antibody, and the binding indicates a positive ELISA test.(Elham Sheikhzadeh, 2020)

In this study, the main aim was to evaluate an immune system of people who are already got the vaccine or infected previously, throw measurement of antibody titers and serotypes in SARS- COVID-19 vaccinated human serum, in addition to evaluate the persistent of antibodies against COVID-19 in previously infected individuals. The evaluation is based on ELISA test that is targeting the cloned COVID-19 antigens, mainly (S) protein and (M) protein. The S and M antigens were cloned during this study

and they were evaluated for their usefulness in ELISA tets to detect IgG or IgM antibodies against COVID-19 antigens.

1.1 Scope of work

This thesis work include two groups of subjects were recruited in this study: Group one is sera of previously infected individuals or vaccinated individuals who already had been taken vaccines against corona virus disease, which contains subjects from both males and females of different ages who don't suffer currently from COVID-19 infection, while Group two is a normal individuals who didn't undergone to any previous vaccination against corona virus, which contains subjects from both males and females of different ages.

1.2 Thesis organization

This thesis is divided into five chapters including:

Introduction, Literature review, Methodology: Method and Materials, Results, Discussion and Future work with Conclusion, as follows:

Chapter one contains Introduction and the general overview, objectives, and the scope of work.

Chapter two contains the deep meaning of corona virus and previous studies.

Chapter three introduces materials and methods that used in our study thesis.

Chapter four talk about the results of evaluation immune system by ELISA method.

Chapter five presents the discussion, conclusion, suggestion for future work developing, references and appendices.

1.3 Problem statement

In Palestine, COVID 19 infect many people very fast. It spread in high manner and vaccination was used in many places in the world to stop its spread. The COVID-19 vaccines primarily use immunological and molecular methods for viruses combating.

Immunizations are crucial to the eradication of infections. ELISA technology, generally yields reliable findings quickly and has strong specificity as well as sensitivity. It is capable of identifying COVID-19 viral antigens or antibodies within respiratory, blood, urine, also saliva, & stool samples.

Therefore, in order to tackle the Covid-19 virus in Palestine, it is necessary to use the most effectives vaccines that are simple, affordable, and can produce a long term humoral immunity.

The Evaluation of vaccine effectiveness was based on ELSA test that considered as easily doing approach, can identify serum antibodies, which is helpful in examining patient immunity via measuring antibody titers, and hence vaccine response, especially in case of herd immunity.

1.4 Study significance

The worldwide COVID-19 epidemic is currently an extremely significant and hazardous world medical care issue owing to the quick spread for SARS-CoV-2 from one to another, which has resulted in 311 million cases worldwide as well as over 473 thousands patients in Palestine.

According to the World Health Organization's every day COVID-19 Condition Documents, there have been over 5.5 million deaths from COVID-19 globally and nearly 5000 fatalities were recorded in Palestine. Scenario of the Coronavirus Infection Emergency (COVID-19). Accessible via the internet (viewed on June 11, 2022). Compared to other different coronaviruses like SARS and MERS, SARS-CoV-2 spreads more quickly, so that as of right now, there are no authorized medications regarding its treatment. Early detection is therefore essential to stop the disease from spreading widely.

1.5 Study aim and objectives:

The main aim of this study is to evaluate an immune system of Palestinian people who are already got vaccination previously against COVID-19, the specific objectives can be summarized into the following points:

1- To study the effectiveness of COVID-19 vaccination among patients those already has been vaccinated by evaluation of antibody titers, via measurement of COVID-19 antibody titers in human being serum.

2- To measurement and evaluate IgG and IgM antibodies against COVID-19 in sera of vaccinated human being.

3- To compare two groups' results between normal individuals those are not involved in vaccination as a control with previous vaccinated people based on sensitivity and specificity

1.6 Ethical consideration

An ethical approval was submitted to Al Quds University Medical laboratory science research committee for approval. The permission to conduct the study was obtained from the Palestinian Ministry Of Health. Also all patients' consents in this study has been obtained after notifying them about their rights to refuse, participate or withdraw at any time.

Chapter Two

Literature Review

2.1 History

Epidemics of coronaviruses have occurred throughout the last two decades, including MERS in 2012, SARS-CoV-1 in 2003, and SARS-COV-2 in last year 2019. The mortality rates for SARS-CoV, MERS-CoV, and SARS-CoV-2 are 10%, 37%, and 5%, resepectively, and can induce serious signs as well as dying (Huang, 2020).

Although coronavirus infections in humans have been documented since the 1960s, the virus's capacity to trigger deadly epidemics has just recently come to light. The *Coronaviridae* family, includes SARS-CoV-2 (Zhou, 2021). The coronavirus was initially identified as the cause of the common cold in 1960, when the first cases of infection in humans were reported (McIntosh 1967).

The 2003 outbreak of Severe Acute Respiratory Syndrome (SARS) in China resulted in around eight hundred deaths while participating 8400 cases, making it the first epidemic of the twenty-first century.(Ortega, 2020)

Afterwards the rapid dissemination of COVID-19, which was initially appeared on December 31, 2019, at Wuhan, China, the history of corona viruses continued to late 2019. In year 2020 it has been declared a public health emergency due to the outbreak of disease (Wu YC 2020). Since then, the infection has been spreading quickly over the world, leading the World Health Organization (WHO) to declare the illness a "global health danger." The Center for Disease Control (CDC) recorded COVID-19 within each country in the world. COVID-19 is a reported disease by the CDC. With almost 375,000 recorded deaths in 2020, COVID-19 was the third leading cause of mortality, following malignancies and diseases of heart (Fein, 2021). SARS CoV2, is responsible for pandemic illness and acute respiratory syndrome. Fever, exhaustion, dry cough, upper chest discomfort, sporadic diarrhea, & dyspnea are its main symptoms. They experience lymphopenia, pneumonia, and pulmonary opaqueness of ground glass in chest CT scans. (Taefehshokr, 2020). The first wave of COVID-19 caused up to one million cases deaths worldwide, which prompted the United Kingdom and several other governments to enforce the stay at home restriction in March and April, respectively. (Ioannidis, 2020)

Other strains of corovaviruses such as SARS-CoV and MERS-CoV have positive-sense RNA genomes of 27.9 and 30.1 kb, subsequently, and belong to the *Betacoronavirus* genus of the *Coronaviridae* family (de Wit, 2016)

2.2 Coronavirus Disease: SARS-CoV-2 Biology

The virus appears in ellipsoidal form in the shape having typical diameters of about 100 nm along its short, middle, also tall axes, subsequently (Zhu, 2020). It also exhibits a distinctive like crown in the shape in architecture morphology. Spike proteins give the virus its distinctive look, whereas having ten times fewer copies compared with an influenza virus, it is similar in size like HIV. This virus is construct from Y-formed spiky pairs and have two heads and one stem in a tiny population.

Each particle of the virus's RNA contains nearly about from 30 to 35 ribonucleoproteins (RNPs), which are closely packed together in the lumen. Around to 80 nm in a diameter lumen, a single-stranded positive-sense RNA genome of SARS-CoV-2 has 15 open reading frames in its whole genome which is 29.8 kilobases in

length. (Yao, 2020). So, there are a total of twenty nine proteins are encoded by 15 open reading frames in SARS-CoV-2 genome. In contrast, ORF2 encodes a spike protein and also ORF4 a membrane protein, ORF9 a nucleocapcid, with the balance of ORFs generate accessory proteins, according to (Chukwudozie, 2020)

ORF1a and ORF1ab form polyproteins which include 16 non-structural proteins. The factors that me play a role how long the period that a virus may live is determined by the ability to enter a cell, develop within a cell, replicate in a host cell, and avoid immune cells. To control the spread of SARS-CoV-2, researchers must understand the functions of these 29 proteins (Chukwudozie, 2020) In addition to the lack of knowledge about SARS-COV2, RNA viruses have a higher capacity to mutations rather than DNA viruses, which makes it much difficult to regulate its epidemic and related diagnosis and hence its treatment. (Wang and Yu 2004)

According to investigations, SARS-CoV-2 belongs to the exact similar species as SARS-CoV. It is referred to as novel coronavirus or SARS-CoV-2 since it is diinct to a zoonotic coronaviruses MERS-CoV also SARS-CoV-1, both of which were transmitted to people prior at a 21st century.

2.3 Taxonomy

According to The International Committee on Taxonomy of Viruses, Table 1 presents the Taxonomy of SARS CoV2 virus.

Table 1 it presents the Taxonomy of SARS CoV2 virus	
Category	Coronavirus
Realm	Riboviria
Order	Nidovirales
Suborder	Cornidovirineae
Family	Coronaviridae
Subfamily	Orthocoronavirinae
Genus	Betacoronavirus
Subgenus	Sabrecovirus
Species	Severe acute respiratory syndrome related coronavirus
Individuum	SARS CoV2

2.4 Structure and genotype of Coronavirus

The coronaviruses genome is about 32 kb in size, have the largest and longest sizes of all the positive RNA viruses. SARS-COV2 consists of 4 structural proteins that cooperate with the +RNA genome and envelope to create the virion, in addition to the 16 non-structural proteins necessary for RNA replication. The 3 proteins membrane, spike, and envelope as well as the protein Nucleocapcid are essential for the development of the viral particles that result in viral infection, in addition to packaging the RNA genome. (Chukwudozie, 2020)

2.5 Coronavirus transmission across species

It is generally known that interspecies transmission plays a essential role in the development of viral diseases. For example, viruses from wildlife hosts have caused significant diseases such as influenza, Ebola fever, and severe acute respiratory syndrome (SARS) in humans (Parrish, 2008).

The accessibility of susceptible and permissive host cells in the host, the presence of host cells with the required receptor for viral entry, the permissiveness of these host cells to allow the virus to replicate and complete their replication cycle, and the inability of the host cells' innate immune response to resist are generally many main factors that determine the success of cross-species transmission of a particular virus. (Hulswit 2016).

Today, coronaviruses are known as one of the most rapidly evolving viruses due to their high genomic nucleotide substitution and recombination rates (Lim, 2016). SARS viruses, for example, have the capacity to be directly transmitted from animals to humans (Rosenberg, 2015).

2.6 Replicative cycle of coronaviruses

2.6.1 Viral entry and membrane fusion

The first step in viral replication cycle and hence in its infection to host cells starts by viral attachment via its a spike proteins to host specific receptors, in human it is ACE2 (Angiotensin converting enzyme 2). The infection is done if this process is initiated by the virus. Binding of the virus particles to the cellular receptors which leads to viral entry followed by fusion of the viral and host cellular membranes (Figure 1). The coronavirus S proteins' S1 subunit (domain) is crucial for mediating the S protein's interaction to the host receptor. The vast host spectrum for this virus family is partially explained by the S1 component (Walls, 2017).

The membrane fusion process enables a viral genetic material to be uncoated—a mechanism that causes the viral genetic material accessible to translation in the cytoplasm of the host cell. The transmembrane spike (S) glycoprotein, which promotes receptor binding and fusing between viral and host cell membranes, it is facilitates coronavirus penetration. The spectrum of host species and tissue tropism are mostly determined by the interaction of the S protein with the cellular receptor (Masters, 2006). One of the most significant characteristics of coronaviruses is their variety for receptor utilization, which exhibits complicated patterns when it comes to receptor detection & recognition (Li, 2016). Table 2 provides a list of the coronavirus cellular receptors for humans.

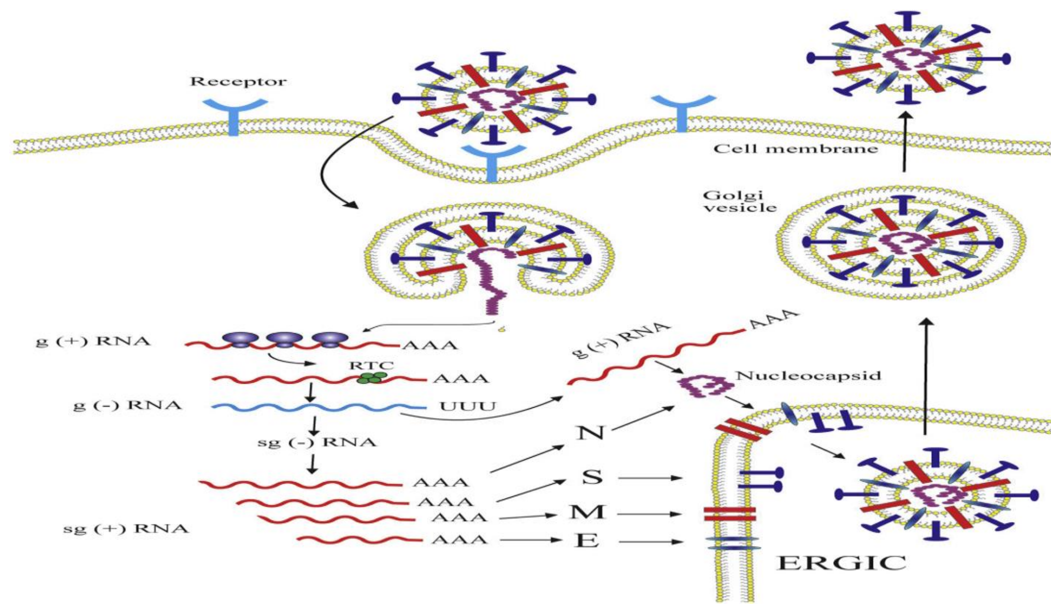

Figure 1 Coronavirus life cycle diagram

Table 2. Receptor of human pathogenic coronaviruses.		
CoV-229E	Human aminopeptidase N (CD13)	Yeager et al. (1992)
CoV-NL63	Heparan sulfate proteoglycan	Milewska et al. (2014)
CoV-HKU1	9-O-acetylated sialicacid (9-O-Ac-Sia)	Huang et al. (2015)
CoV-OC43	9-O-Acetylated sialic acid (9-O-Ac-Sia)	Vlasak et al. (1988)
SARS-CoV	Angiotensin-converting enzyme 2 (ACE2)	Li et al. (2003)
MERS-CoV	Dipeptidyl peptidase 4 (DPP4; CD26)	Raj et al. (2013)
SARS-CoV-2	Angiotensin-converting enzyme 2 (ACE2)	Zhou et al. (2020)

2.6.2 Replication of coronavirus genome

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 commanding the host ribosome machinery. The plus ssRNA is replicated into dsRNA

by the expressed viral RNA polymerase. Nucleocapsid (N), spike (S), membrane (M), 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).

Complex methods involving numerous proteins encoded by both the viral and host cell genomes are used by coronaviruses to replicate. RNA viral genomes produce several proteins such as: The RNA-dependent RNA polymerase (RdRp), RNA helicase, chymotrypsin- and papain-like proteases, and metal binding proteins. All of the genes that code for these proteins in coronavirus genomes are found in the ORF1. Additionally, during the course of their replication cycle, viruses utilize host cellular proteins and enzymes for a variety of processes, including cell attachment and entrance, the start and control of RNA replication and transcription, protein synthesis, and the construction of offspring virions (Shi, 2005).

The viral RNA genome is released and uncoated, and the genomic replication cycle begins shortly after receptor binding and membrane fusion events are completed. A coronavirus replicates its genome by synthesizing a complementary negative (–)-strand RNA using the genomic RNA as a template, similar to all other positive (+)-stranded RNA viruses. Table 3: shows most important coronaviruses that infect human.

Table 3. Human pathogenic coronaviruses.

Virus	Genus	Natural Host	Year of discovery	Symptoms
HCoV-229E	α-coronavirus	Bats	1966	Mild respiratory tract infections
HCoV-NL63	α-coronavirus	Bats	2004	Mild respiratory tract infections
HCoV-OC43	β-coronavirus	Rodents	1967	Mild respiratory tract infections
HCoV-HKU1	β-coronavirus	Rodents	2005	Pneumonia
SARS-CoV	β-coronavirus	Bats	2003	Severe acute respiratory syndrome, 10% fatality rate
MERS-CoV	β-coronavirus	Bats	2003	Severe acute respiratory syndrome, 37% fatality rate
SARS-CoV-2	β-coronavirus	Bats	2019	Severe acute respiratory syndrome, 3.7% fatality rate

2.6.3 Virions assembly and budding

The place where coronaviruses assemble their virions is one of their distinguishing characteristics. Virions assembly occurs at the plasma membrane of the host cell for the majority of enveloped viruses. However, for coronaviruses, the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) is where virions budding and assembly take place (Walls, 2017). As a result, ERGIC serves as the source of the membrane envelope for coronaviruses (Ujike, 2015). The three membrane (enveloped) proteins need to be kept close to the ERGIC for effective coronavirus virion formation. In reality, intracellular trafficking signals on the M, E, and S proteins direct these structural proteins to the budding site, where they accumulate.

The M proteins are primarily responsible for mediating the protein-protein interactions necessary for coronavirus assembly. According to (Woo, 2019), the coronavirus packaging signal (PS), a regulatory element encoded within the viral RNA, is responsible for packing the viral genome into the ribonucleocapsid.

In the presence of the N protein or the E protein, the M protein can form virus-like particles (VLPs), indicating that they have a crucial role in the virions assembly (Tseng, 2010). It is likely that specific interactions between the M protein and the S, N, and E proteins are what cause the coronavirus to assemble. The coronavirus structural proteins S, E, and M must be inserted into the endoplasmic reticulum (ER) in order to produce mature virions. Following that, the structural proteins engage in interactions with the encapsidated viral genomes and come together by budding to form mature coronavirus particles (Fehr, 2015). In smooth-walled vesicles after assembly, the progeny virions are transported to the cell surface and discharged into the extracellular space by exocytosis or cell lysis (Orenstein, 2008).

2.7 Molecular characteristics of SARS-CoV-2

2.7.1 SARS-CoV-2 ribonucleoprotein variants

Coronavirus RNA variants refer to the different versions of the SARS-CoV-2 virus, , that emerge as the virus replicates and mutates. Since coronaviruses, like SARS-CoV-2, have RNA genomes, they are prone to mutations during replication. These mutations

can lead to changes in the virus's structure, particularly in the spike protein, which can affect how the virus infects cells, how the immune system responds, and how effective treatments or vaccines are. Some variants, such as Alpha, Beta, Delta, and Omicron, have gained global attention due to their increased transmissibility, ability to evade immunity, or changes in disease severity. The rapid evolution of these variants has made monitoring and research crucial in controlling the pandemic and improving public health responses. It is important to know that human disease has been linked to the β-coronavirus and α-coronavirus. In contrast to animal sickness has been linked to members of the genera δ-coronavirus and γ-coronavirus (Masters, 2006).
The Alpha, Beta, and Delta variants of COVID-19 are some of the earliest and most notable strains of the SARS-CoV-2 virus that gained attention due to their mutations, transmissibility, and impact on public health. Here's a breakdown of each:

Alpha Variant (B.1.1.7): was first identified in the UK in September 2020 and quickly spread to other parts of the world. It had several mutations in the spike protein, including the *N501Y* mutation, which appeared to make the virus more infectious by allowing it to bind more tightly to human cells. The Alpha variant was associated with increased transmissibility, meaning it spread more easily compared to the original strain of SARS-CoV-2. It was also linked to potentially higher rates of hospitalization and severity of illness, though the available vaccines remained effective against it.

Beta Variant (B.1.351): was first detected in South Africa in May 2020. This variant contained mutations such as *E484K* and *K417N* in the spike protein, which raised concerns about its ability to partially evade the immune system, including some of the neutralizing antibodies generated by vaccines or past infection. The Beta variant was more likely to cause reinfections and may have been less susceptible to certain vaccines and treatments.

Delta Variant (B.1.617.2): was first identified in India in late 2020 and became a dominant strain globally by mid-2021. The Delta variant carried several mutations in the spike protein, including *L452R* and *T478K*, that increased its ability to bind more effectively to human cells, making it highly transmissible. It was estimated to be 50-60% more contagious than the Alpha variant. Delta was associated with higher viral loads, leading to increased transmission, even among vaccinated individuals.

2.7.2 Spike (S) protein

The coronavirus spike (S) protein is a large glycosylated transmembrane protein ranging from about 1162 to 1452 amino acid residues. Monomers of the S protein, prior to glycosylation, are 128–160 kDa, but molecular masses of the glycosylated forms of the full-length monomer are 150–200 kDa. The coronavirus got its designation because of how their fringe mimics solar corona (Masters, 2006). Figure 2, Shows a schematic representation of a coronavirus virion. The spike (S) glycoprotein spicules of a host cell-derived bilayer of a lipids along with the membrane (M) and envelope (E) transmembrane protein molecules, giving a virion characteristic look. Under the towering S protein spikes, the haemagglutinin esterase (HE) generates smaller spikes. The nucleocapsid phosphoprotein (N) and the positive-sense viral genomic RNA collaborate to generate the ribonucleoprotein, which has the helical form of the structure (Masters, 2006).

This Figure describes the structure of the SARS CoV2 virus and its major components, taken from (Yao et al., 2020).

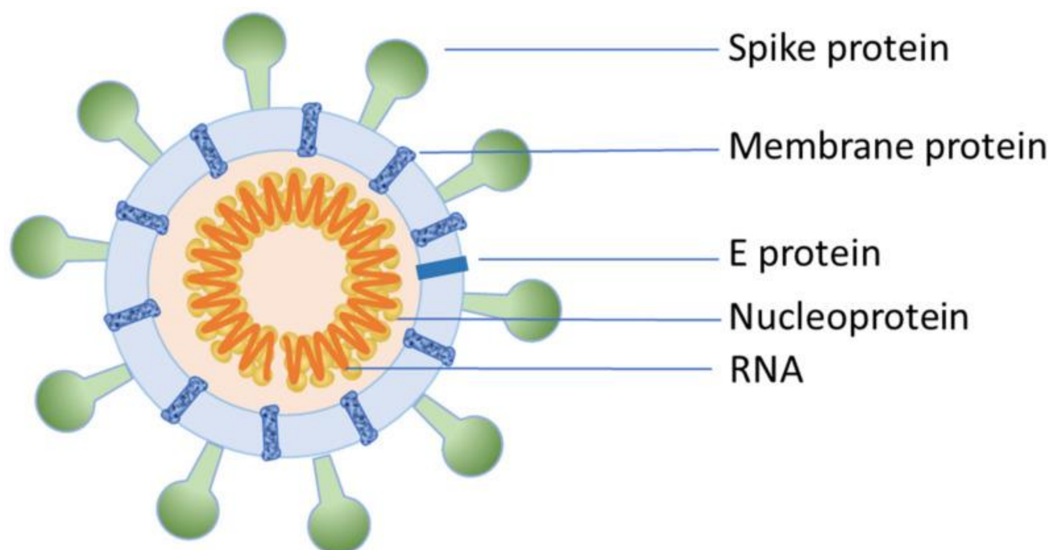

Figure 2 The structure of the SARS CoV2 virus and its major components

The S protein is the most outward envelope protein of the coronaviruses. The S glycoprotein plays critical roles in mediating virus attachment to the host cell receptors and facilitating fusion between viral and host cell membranes. In addition, it is the primary determinant of the coronavirus tropism. Spike protein that mainly binds cell

receptors (ACE2) is widely conserved in all human coronaviruses and is involved in receptor recognition, viral binding, and host cell entry. It is one of the most important candidates for the COVID-19 vaccine and clinical research due to its essential functions (Petrosillo et al., 2020).

2.7.3 Membrane (M) protein

The membrane (M) glycoprotein is the most abundant envelope protein of coronaviruses playing critical roles in the virion assembly through M-M, M-spike (S), and M-nucleocapsid (N) protein interactions (Arndt, 2020). Generally, its length is 217–230 amino acids. It is a triple-spanning membrane protein with a short amino-terminal domain located on the exodomain of the virus (in the virion exterior, equivalent to the lumen of intracellular organelles) and a long carboxy-terminal domain in the endodomain of the virion (in the virion interior, equivalent to the cytoplasmic space of intracellular membranes) (Masters, 2006) (de Haan, 1999)

2.7.4 Envelope (E) protein

The envelope (E) protein is a small integral membrane polypeptide, ranging from 76 to 109 amino acid residues with molecular weight of 8.4–12 kDa. The E protein plays important roles in a number of aspects of the coronavirus replication cycle, such as assembly, budding, envelope formation, and pathogenesis. Interestingly, although the protein is highly expressed inside the infected cells, only a small portion of the protein is incorporated into the viral envelope. Consequently, the protein is only a small constituent of the virus particle. hydrophilic carboxy terminus (Masters, 2006; McBride, 2012). Due to its small size and limited quantity, the E protein was identified much later compared to the other coronavirus structural proteins. Its primary and secondary structure indicates that the E protein has a short hydrophobic N terminus of 7–12 amino acid residues, followed by a transmembrane domain (TMD) of 25 amino acids.

2.7.5 Nucleocapsid (N) protein

The coronavirus nucleocapsid (N) protein, which makes up a portion of the helical nucleocapsid, is a structural phosphoprotein weighing 43–46 kDa. The primary role of the N protein is to package the viral genome into an RNP particle, which protects the genomic RNA and allows it to be incorporated into a functional virion. The genomic RNA is thought to be bound by the N protein in a beads on a string manner. Additionally, it interacts with the viral membrane protein as the virion is being assembled, which is crucial for increasing the speed of virus transcription and assembly. Following production, the N protein experiences rapid phosphorylation (Lu et al., 2011).

2.7.6 Accessory proteins

All coronavirus genomes have auxiliary genes scattered throughout the replicase, S, E, M, and N canonical genes. The number of auxiliary genes varies from one (HCoV-NL63) to eight (SARS-CoV). These auxiliary proteins are not necessary for coronavirus replication, but they might provide the coronaviruses with biological advantages in the surroundings of the infected host cells. Certain auxiliary proteins have been demonstrated to play roles in the interaction between viruses and their hosts and appear to play a role in viral infections Table 4 lists the auxiliary proteins that the coronaviruses which infect people encode (Masters, 2006; Wang, 2020)

According to studies on SARS-CoV, several accessory proteins can affect interferon signaling pathways with the production for pro-inflammatory cytokines (Liu, 2014; Masters, 2006; McBride, 2012)

Table 4. Accessory proteins of human coronaviruses

Virus	Accessory genes (Proteins)
HCoV-229E	[rep]-[S]-4a,4b-[E]-[M]-[N]
HCoV-NL63	[rep]-[S]-3-[E]-[M]-[N]
HCoV-HKU1	[rep]-2(HE)-[S]-4-[E]-[M]-[N], 7b(I)
HCoV-OC43	[rep]-2a-2b (HE)-[S]-5 (12.9k)-[E]-[M]-[N], 7b(I)
SARS-CoV	[rep]-[S]-3a,3b-[E]-[M]-6-7a,7b-8a,8b-[N], 9b(I)
MERS-CoV	rep]-[S]-3-4a,4b-5-[E]-[M]-8b-[N]
SARS-CoV-2	[rep]-[S]-3a,3b [E]-[M]-6-7a,7b-8b-[N],9b,10

2.7.7 Genome

A single-stranded RNA molecule with a positive sense, or +ssRNA, makes up the coronavirus genome. This ssRNA is comparable to mRNA molecules. Its structure, which includes 3'poly-adenine tails and also the 5'caps, is similar to that of the majority of an eukaryotic mRNAs. The astonishingly huge size of a genome for a coronavirus which ranges from size of 26 kb and reach to 32 kb in size, is one of its distinguishing characteristics. In order to comparison, it is notable that this is roughly 3 times the size of the genomes of flaviviruses or alphaviruses and also its 4 times to the size of the genetic material of picornaviruses (Chukwudozie, 2020). In fact, the genetic material for coronavirus is consider to be the biggest viral genomic RNAs that are currently known. A multiple of open reading frames ORFs are present in the genetic material of corona virus (Figure 3), and these ORFs encode structural and non-structural proteins (Setianingsih, 2019). In addition to the large spectrum of accessory proteins that vary in number, the sequence composition of coronaviruses (Chen, 2020; Masters, 2006; Setianingsih, 2019).

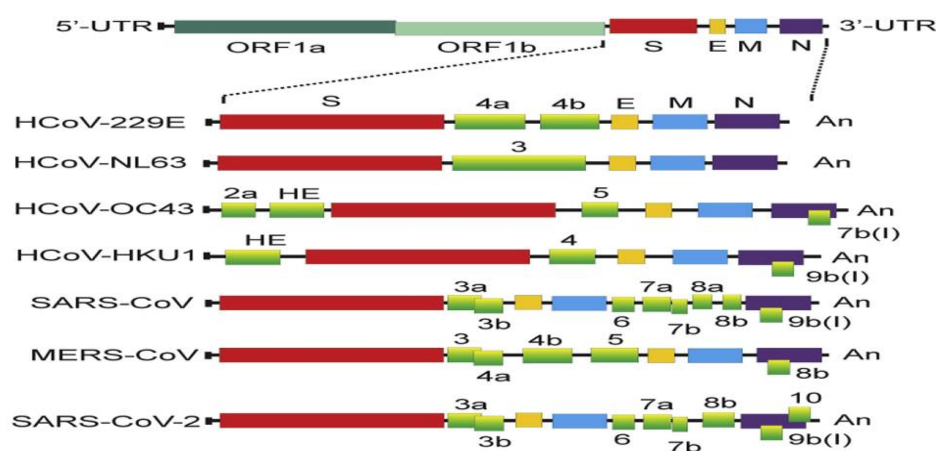

Figure 3 Schematic illustration of the human coronavirus genomes

The genetic organization of each coronavirus is represented by a bar. A comparison of the genomic regions or open-reading frames (ORFs) of human coronaviruses The non-structural proteins translated from ORF 1a and ORF 1b and accessory proteins are indicated, together with the structural proteins, including spike (S), envelope (E),

membrane (M), and nucleocapsid (N) proteins (Chen, 2020; Masters, 2006; Wang, 2020).

2.8 Immunity against COVID-19

Herd immunity is a form of indirect immunity that arises when a substantial portion of the population becomes immune to an infection through infection or vaccination, hence preventing the virus's spread. The adaptive immune system's B cells and antibodies, which attack specific infections, are present (Bulut, 2020). Physical barriers: the outmost tissue walls that may prevent viruses from invade by using defensins which in result alarming neighboring cells from the danger that will infect cells (Koltman, 2006)**.** The end result is to block invasion and hide receptors via inhibition of gene expression and release of RNAase to cytosol.

2.9 Pathogenesis

Throw using models of an animal make easily both of pathophysiology & therapy, a prior research on lab mice aided understanding both of SARS virus and also MERS one (Gretebeck, 2015)

Similar to receptor of SARS-COV as well as SARS-COV2, the angiotensin converting enzyme 2 (ACE2) receptors that trigger the infection are expressed in macrophages, respiratory epithelium, alveolar monocytes, and also in vascular endothelium (Ni, 2020).

According to Chapel, Haeney et al. (2013), coronavirus entering relies on a viral spike (S) proteins attaching on cellular receptors while S protein priming via host cell proteases. Spike protein (S) is required for the start of cell host entry and confers increased virulence on the virus when it recognizes lung alveolar cells. As of this writing, in the middle of the third wave, numerous researches have confirmed the presence of receptor ACE2 in various tissues, including small intestine enterocytes, arterial and venous endothelial cells, as well as arterial smooth muscle cells (Cao, 2020)

2.10 Vaccination

Patients experience a range of challenges as a result of COVID-19 owing to currently is no efficient therapy for the disease. A number of life-support medications will be needed in order to survive and reduce the losses caused by the disease. Extracorporeal membrane oxygenation (ECMO) and antiviral medications are two instances of these therapies. Numerous trials and other investigations have shown that there is no specific treatment for COVID-19; instead, patients are given a variety of medications and control strategies (Viner, 2020).

The vaccination boosts the immune response and prepares the body's defenses to detect and eradicate the virus when it comes into exposure to it. Nucleic acids (DNA and RNA), protein components, live attenuated viruses, inactivated viruses, and viral vectors have all been utilized in the development of both replicating and non-replicating vaccines (Khade, 2021)

Vaccines types available in Palestine:

- Sputnik Russian vaccine
- Moderna COVID-19 Vaccine
- Pfizer-BioNTech COVID-19 Vaccine
- AstraZeneca vaccine
- Sinopharm Vaccine

2.11 Treatment

Famous Therapies used in treatments of corona viruses (Forchette, 2021)

- Remdesivir
- Molnupiravir
- Plitidepsin
- Zotatifin
- Ivermectin

Last update April, 8, 2020 based on COVID-19 Treatment Guidelines Panel. Coronavirus Disease 2019 (COVID-19) Treatment Guidelines. National Institutes of Health. Available at nih/gov treatment guideline.

2.12 Epidemiology of COVD-19 world wide

The emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) late December 2019 in Wuhan, China, marked the third introduction of a highly pathogenic coronavirus into the human population in the twenty-first century. The constant spillover of coronaviruses from natural hosts to humans has been linked to human activities and other factors. SARS-CoV, MERS-CoV, and SARS-CoV-2 are more likely to spread through other zoonotic reservoirs, with the occasional breakout in the susceptible human population, perhaps through an intermediary host animal (Su, 2016).The new coronavirus has a high incidence of from person to person transmission and can cause a variety of clinical symptoms in those who are infected (Yao, 2020)

When a person is in close proximity (less than 1 meter) to someone who is coughing or sneezing, SARS-CoV-2 can spread by droplets. The virus may spread through the mucosae (mouth, nose, and eyes) or conjunctiva of an infected person. Viral transmission through the conjunctiva is more infrequent, despite mucosal transmission being the most frequent (Peng, 2020).

2.13 Palestine's COVD-19 medical epidemiology

When 7 Palestinians tested positively to SARS-CoV-2 at Bethlehem dated the fourth of March in 2020, a 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. (https://www.emro.who.int/). The state of emergency has been declared in effect for one more month. The restrictions were lifted on May 25th, 2020, due to a drop in incidences and a decrease in the rate of positive tests among Palestinian workers leaving Israeli territory.(https://www.emro.who.int/)

2.14 Diagnosis of Corona virus

Individuals experience varied clinical manifestations of SARS-CoV-2, and this heterogeneity in local prevalence makes it difficult to identify COVID-19. The patient history, clinical condition, and laboratory testing all play a role in the COVID-19 diagnosis For individuals with suspected COVID-19, nasopharyngeal, sputum, bronchial aspirate, and blood are usually recommended (Rai, 2021). To avoid any

misdiagnosis of infection with other respiratory system viruses, medical condition, disease history, signs, and symptoms are typically used in conjunction with laboratory tests. Patients are segregated, and each one has a chest X-ray, blood sample, and nasopharyngeal specimen obtained (Rai, 2021). In addition to RT-PCR technique, serological and immunoassay test are also recommended a supplement. There are a wide variety of serological techniques, including affinity chromatography, chemiluminescence assays (CLIA), enzyme-linked immunosorbent assays (ELISA), western blotting (WB), immune-fluorescence assays (IFA), and protein microarrays (Padoan et al. 2020). Serological diagnostic methods (such as ELISA and immunochromatography) that are combined with RT-PCR to develop the diagnosis accuracy assurance and sensitivity (Guo et al. 2020).

Serum antibodies screening testing is used to detect IgM and IgG antibodies against COVID-19 virus in the body fluid. Antibodies are usually formed a few days after infection with early increase in the IgM level followed by class switching IgG, then IgA (Roy et al. 2020), uses of enzyme - linked immunosorbent assay (ELIZA) as a quantitative method to detect the titer of IgG and IgM antibodies that provide a robust mean to quantify the level of antibodies which can be a key to identified the immunity threshold in the SARS-COV-2 infected patient (Roy et al. 2020), virus neutralization is considered as the gold standard method for a serological test (Sapkal et al. 2020). The determination of COVID-19 is highly dependent on the detection of IgM or IgG antibodies Specific to various viral antigen, such as: the spike glycoprotein (S1 and S2 subunits, and a nucleocapsid protein, these antigens are basically used in enzyme-linked immunosorbent assay (ELISA) which has high throughput capacity (Liu et al. 2020), and providing quantitative exposure and immune response (Roy et al. 2020),and immune chromatographic lateral follow immune assay, neutralization bio-assay, and specific chemo-sensors (Carter et al. 2020). Also Western Blot Assay can be used for Detection of Antibodies against Coronavirus.

Chapter Three

Material and Methods

3.1 Samples

Serum samples were collected from 110 individuals who are visiting Beit Jala Governmental Hospital for different examination purposes, from these sample 91 were sera for vaccinated individuals and 19 sera for normal non-vaccinated. sample collection was random with no preferences to age. The collected samples included males and females and all were above 20 years old. Those individuals varied in their COVID-19 vaccination status: 24 individuals have received single vaccination dose, 41 individuals whom received two vaccinations doses, and 27 individuals were vaccinated with three doses. The collected sera included 18 samples for individuals whom were not received COVID-19 vaccination.

About 5 ml blood sample was collected from which the serum was isolated following standard laboratory methods for serum collection, the collected serum samples were stored at -20 ^{o}C until they were used in this research. Samples were collected mainly

from two categories (vaccinated and non-vaccinated individuals), all previously infected individuals were mainly excluded from this study. Samples were collected mainly from adults whether females or males.
An ethical approval for samples collection was obtained from Al-Quds University central ethical committee. All participants were informed about the purpose of the study and signed a consent form.

3.2 Cloning of SARS CoV-2 Surface and Membrane genes

From previous work in our immunology lab, we successfully cloned SARS CoV-2 spike (S) protein and membrane (M) protein genes. In brief we amplified the S and M DNA gene segments from Cov-2 cDNA (already prepared for diagnostic proposes in the hospital laboratory) taken from infected individuals and the PCR product, was cloned in an expression plasmid vector named (pET-28a). The plasmid was transformed in bacterial cells (*Escherichia coli* BL21 cells) based on $CaCl_2$ and $MgCl_2$ transformation protocols. Single bacterial colonies were isolated for both S and M genes and were tested for harboring the exact nucleotide sequence of these two genes (Zhu 2020).

3.3 Amplification of CoV-2 Surface and Membrane gene segments by Polymerase chain reaction (Quality control test)

Currently and before we start antigen preparation we confirmed that the expression plasmids still have the S and M DNA genes. For this purpose we used two PCR systems one for S and the other for M DNA gene amplification, in each PCR system we used the same primers that were already used to amplify the S and M Cov-2 genes (Table 5).

The PCR reactions were performed using ready mix Taq DNA polymerase (Syntezza, Jerusalme). For each PCR reaction a mixture of a total volume 25 µl was prepared, the mixture includes 1 µl of (a total of 20 pmoles) of forward and reverse primers of each PCR system used to amplify Membrane and Spike gene segments together with 3 µl of the recombinant plasmid (pET28), the final volume was completed using double

distilled water. The PCR was performed using MJ-research PCR machine from Bio-Rad. The used program includes denaturation step at 95°C for 5 minutes followed by 35 cycles of (denaturation at 95°C for 30 secnds, annealing at 57°C for 30 seconds, and an elongation step at 72°C for 1 minutes), this was followed by 1 min at 72°C as complete extension step.

Table 5: DNA sequence of forward and reverse primers that were used in amplification of membrane and spike cloned DNA segments in pET plasmid.

Gene segment	Primers sequence	Tm (°C)
Membrane	**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	**57**
Spike	**Direct:** GGG GCT CGA GAT GTT TGT TTT TCT TGT TTT ATT GCC ACT AG **Reverse:** GGC GAA GCT TAG GGA GAT CAC GCA CTA AAT T	**57**

3.4 Agarose gel electrophoresis

The PCR products were analyzed on 1.5% agarose gel electrophoresis using 1x TAE buffer (50X TAE running buffer contains (242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (pH 8.0)). The gel was run at 120 volts for 30 minutes and the amplified PCR products (which was expected to be about 700 bp) was measured against a 100 bp ladder (Thermo Scientific, USA).

3.5 Expression and purification of SARS CoV-2 Surface and Membrane proteins

Expression of large amount of membrane and spike proteins was required for both ELISA and Western-blot analysis. The expression and purification for each used S and M proteins were performed in separately. This was started from single confirmed bacterial colony that have the S or M recombinant plasmid. The single colonies were initially grow for overnight in 5 ml LB media (5 grams of yeast extract, 10 grams peptone, and 10 grams NaCl, all dissolved in 1 Liter of distilled water). Using this

overnight starter culture a 50 ml of LB media was inculcated with 0.5 ml starter bacteria in 500 ml sterile flask, and to the mixture a 30 µl of 100 µg/ml kanamycin antibiotic was added as a selective antibiotic for transfromed bacterial cells. The flask was incubated at 37°C with shaking for 2 hours and this was followed by the addition of 30 µl of 1 M Isopropyl-ß-D-thiogalactoside (IPTG) as an expression inducing reagent was added then the incubation was continued for overnight at 37°C with shaking. On the next day, the flask content was transferred into 15 ml and centrifuged at 3000 rpm for 10 minutes, the pellet was taken and stored with 2ml of PBS buffer (0.24 g KH_2PO_4, 1.78 g Na_2HP_4 , 8 g NaCl) at -20°C.

The collected pellet from each tube was lysed by the addition of 1:1 volume of lysis buffer containing 10mg lysozyme that breaks down the bacterial peptifoglycan cell wall (Lysis buffer: 50 mM NaCl, 0.1M Tris-HCL pH 7.5, 0.05M EDTA, and 0.1% Triton x100). The mixture was incubated at 37°C for 2 to 3 hours, followed by centrifuged at high speed (14,000 rpm) for 10 minutes. The collected supernatant from different preparations for the same protein whether that was M or S proteins were pooled together and protein concentration was measured using UV spectrophotometer at 280nm. Protein concentration was estimated based on the equation (Optical reading at 280nm of 1 O.D equals to 1.1 mg/ml protein) Green (2012).

3.6 Serum testing using ELISA method (Enzyme Linked Immunosorbent Assay (ELISA)

As indicated in the previous section it was possible to prepare large amount of M and S proteins. The prepared recombinant proteins were diluted to 100µg/ml in 1x PBS pH 7.4, this diluted antigen was used to start ELISA assay by coating a 96 wells microtiter plates. For this purpose each well was coated with 100 µl of the diluted M or S proteins, plates were incubated at 4°C for overnight or until their further use. In general the tested sera were two-fold serially diluted starting from 1:50, 1:100, 1:200, and 1:400, and each of the tested samples were examined against both M and S proteins and for both IgG and IgM responses (not all were tested for IgM).

The second step in ELISA method was blocking the coated wells with a 200 µl of blocking buffer (to prevent non-specific binding of tested sera to plastic surfaces) in PBS (this include: 1x PBS, 5% fetal calf serum (FCS), Merck, Sant-louis-USA, 0.05%

Tween-20). Blocking was performed at room temperature for 30 minutes. Then and after the removal of the blocking solution, 100 µl of the serially diluted serum samples were added in different wells plus negative and positive controls, then plates were incubated for two hours at room temperature. Then the tested sera were discarded from the ELISA plates, using 200 µl of washing buffer (1x PBS , 0.05% tween20), the non-binding or non-specifically bound antibodies were eliminated by applying the washing step three times. In order to detect the reacted tested sera a second antibody was added. For the detection of reactive human IgG antibodies, protein-A conjugated to HRP was added in place of the secondary antibody and it was diluted to 1:6000 (as it was recomended by the manufacturer's recommendation on using the secondary antibodies in ELISA test), this was added in a volume of 100 µl to each well containing the tested sera including the blank control wells (Normally 1A and 2A). Plates were incubated at room temperatures for 1 hour, and this was followed with 3 time washing using (1x PBS , 0.05% tween20). Finally, the reacted or bound antibodies to the protein antigens were detected by follow HRP enzymatic assay and this done by the addition of 200 µl of HRP enzyme substrate. The substrate (2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt), simply known as (ABTS), was dissolved in 20 ml of 0.1M tri-sodium citrate buffer (pH5) and 20µl of H_2O_2 , the ABTS concentration was 1mg/ml. Then, the plates were incubated at room temperature for 30 minutes. The results were recorded by reading the absorbance at 405 nm after blanking each plate

3.7 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblotting (Western blot)

The immunoblotting method was performed in order to confirm the reactivity of human positive sera against the expressed Cov-2 membrane and spike proteins. A pool of positive Cov-2 human sera was tested after separating the expressed recombinant proteins on SDS-PAGE. So, this analysis involves first running the polyacrylmide gel electrophoresis, followed by proteins transfer to nitrocellulose membranes and lastly the detection of reactive proteins by Western blot analysis. The detailed procedures for each step are described in the below paragraphs.

3. 7.1 SDS-PAGE Gel preparation

This started by Mini-PROTEAN casting stand and frame (BIO-RAD) were assembled. For gel preparation, two gels were prepared, resolving and stacking gels. The component of the 10% resolving or running gel were (4.7ml of double distilled water, 2.5 of 40% acrylamide, 2.6 of 1.5M Tris-HCl, pH 8.8 and 100 □l from 10% SDS, 100 ml ammonium persulfate, and 10 ml TMED). To ensure that the gel solidified, it was kept at room temperature for around forty-five minutes. Then, the 5% stacking gel was prepared by adding 6.2 ml from double distilled water, 1ml of 30% acrylamide, 2.6 ml of 0.5 M Tris-HCL pH 6.8, 100 □l from 10% SDS, 100 □l from 10% APS and 10 □l from TMED. They were mixed and poured above the resolving gel. After pouring the gel till it reached the glass plates' ends, the comb was placed inserted between the glass plates and left for an additional half hour.

The analyzed protein samples were prepared by mixing 20 □l of the extracted expressed proteins with one volume (20 □l) of loading buffer (Bromophenol 0.004%, 2-mercaptoethanol 10%, Glycerol 20%, SDS 4%, and Tris-HCl 0.125M). Then, the mixture was boiled at 95 C for 10 minutes before loading 15 □l from the sample in each well. The gels was constructed in the gel electrophoresis tank and covered by running buffer (SDS 0.1%, Tris-HCl 25mM and glycine 200mM). The electrophoresis was run at 50 V for about 45 minutes, then at 100 V for another 2 hours.

3.7.2 SDS Gel transfer to Nitrocellulose Membranes

After separation the proteins using SDS-PAGE gel, they were transferred into nitrocellulose filter membrane. The polyacrylamide gel was soaked into 1X transfer buffer (0.025 M Tris-HCl, 0.192 M Glycine, 20% methanol). Then, the gel and the nitrocellulose filter membrane were assembeled together inside the gel holder cassette, then transfer was carried out at 120 V for one hour. The transferred proteins on the NC membranes were reacted with human Cov-2 positive as indicated below.

3.7.3 Western-Blotting

Each membrane was cut into strips. These strips were blocked by 1X PBS, 0.01% Tween-20 containing 5% FCS for 30 minutes. This was followed by the addition of 4 ml to each strip of 1:100 diluted serum samples in 1X PBS, 0.01% Tween-20 containing 5% FCS. Strips were incubated for 2 hours at room temperature. Then strips were

washed 3 times using 1X PBS, 0.01% Tween-20. After that, Protein-A conjugated to HRP was added (1:4,000 diluted in 1X PBS, 0.01% Tween-20 containing 5% FCS) to each strip, and incubated for 1 hour. After incubation, membranes were washed 3 times before the addition of the substrate. The substrate composed from DAB (3,3',4,4'-tetraaminobiphenyl), (10mg DAB, 15□□l H_2O_2, 15ml 50Mm Tis-HCl).

Chapter Four

Results

4.1. Cloned Spike and Membrane genes in Pet-28a plasmid

As it was indicated in material and methods, a previously cloned spike (S) and membrane (M) COVID-19 genes were expressed in Pet-28a expression plasmid vector. Here, it was demonstrated that the S and M genes existed in the plasmids dealt with in this investigation. To do this, a PCR was carried out utilizing the particular primers for the S and M genes, in order to amplify these genes. Results showed that the recombinant plasmids contained both the S and M genes, which have amplicon size about 500-bp and 700-bp, respectively. Figure 4 shows the size of PCR amplicons for the recombinant S and M genes.

4.2. COVID-19 Spike and Membrane gene expression and purification

The XL1-Blue bacteria containing recombinant plasmid pet28a to either S or M gene, these bacteria were used to express these genes to produce the surface and membrane proteins. These proteins used in ELISA and Western-blot analysis. Protein expression and purification was started by 50 ml LB media that was included with 0.5 ml overnight starter Xli-Blue bacteria containing pet28a clone to either spike or membrane. The bacterial cells were treated with lysis buffer and the expressed protein was semi-purified using nickle beads (as it was indicated in material and methods. In general this volume of obtained bacterial cells was suspended in about 2 ml of lysis buffer and the process was repeated twice for each expressed gene in order to enrich the recovered purified proteins. From each clone it was possible to prepare at least 4 ml of 1mg/ml of each S or M proteins that were dissolved in phosphate buffer saline, ready to be used in ELISA or Western-blot analysis and indicated below.

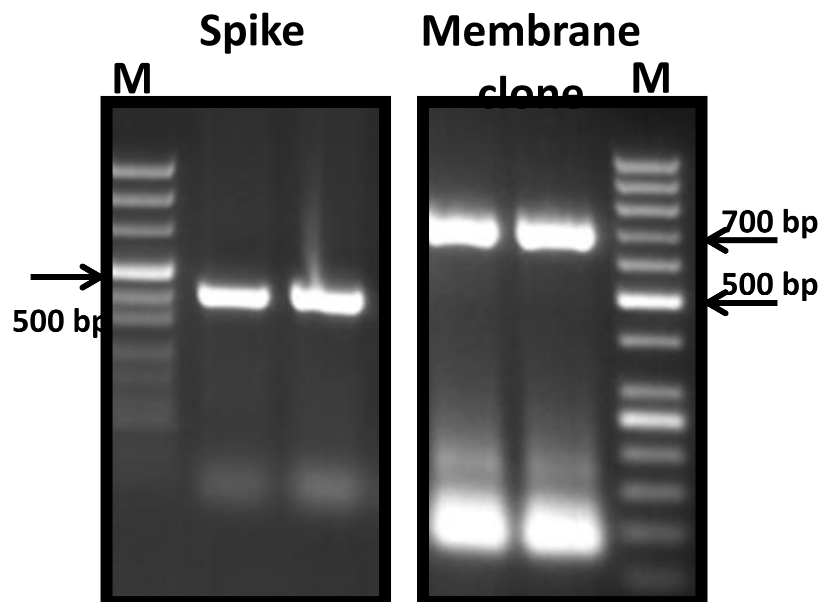

Figure 4 PCR amplification of spike and membrane genes from Pet28a cloning vector. The size of amplified bands was identified as 500-bp for S gene and about 700-bp for M gene. M: DNA size marker

4.3 Reactivity of COVID-19 Spike and Membrane expression proteins to against COVID-19 positive human sera

In this part of the study it was shown that human sera that were previously identified as positive by different serological tests were used as a pool sera (about 10 human samples were mixed together), this pool sera was identified as COVID-19 human positive pool sera and it was later used as in this below indicated Western-blot analysis as well as positive control in the below ELISA analysis. The Western-blot analysis was used to confirm the reactivity of the expressed S and M proteins with known COVID-19 positive sera. Ten human COVID-19 human serum samples were previously identified as positive by different serological tests. These samples were used as a pool serum. The results of SDS-PAGE stained with Commassie-Blue showed many protein bands were still found in the lysate even after its purification using Nickle beads (Figure 5). Figure 6, showed the specific reactivity of the purified expressed S and M proteins with pooled positive human serum. Both the cloned S and M proteins have a molecular weight of about 20-28 kDa. Pooled positive human serum were used as positive control in the ELISA assays.

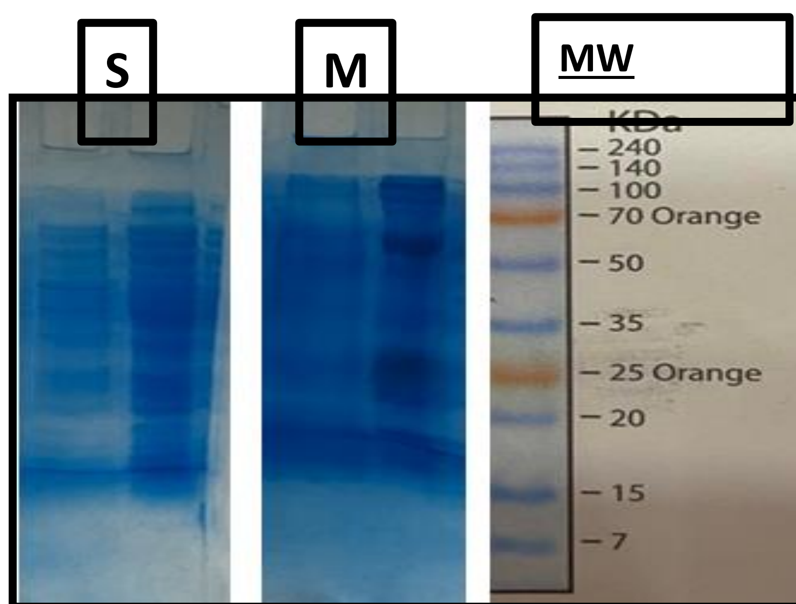

Figure 5 Commassie-Blue staining of Polyacrylamid gel electrophoresis of both spike (S) and membrane (M) expressed and purified proteins. MW: Protein Molecular Weight marker.

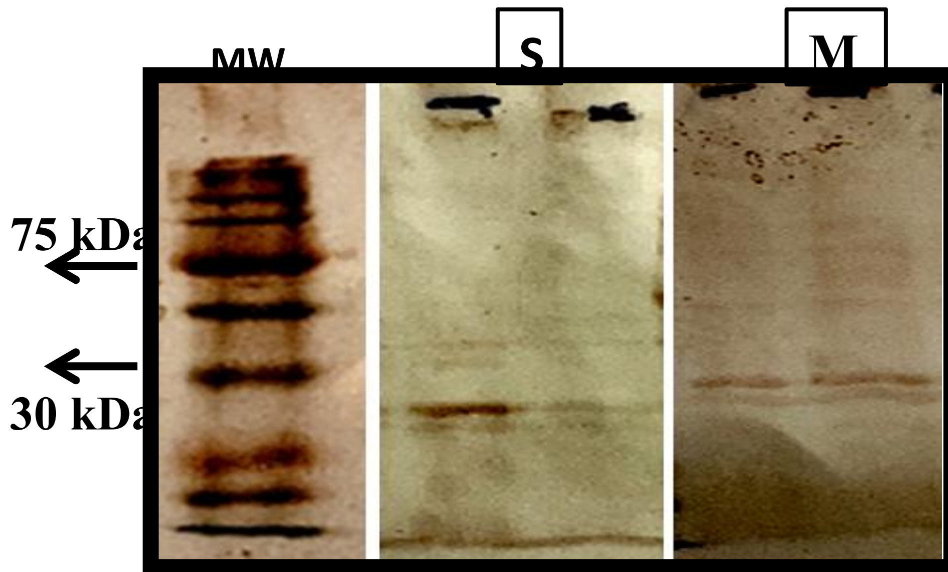

Figure 6 Western-blot analysis using Covid-19 pooled positive human serum against expressed and purified S and M proteins. MW: Protein Molecular Weight marker.

The above immuno-blot results indicate the reactivity of human IgG antibodies (anti-COVID-19) against the S and M semi-purified proteins. The detection of reactivity between the human sera S and M proteins was achieved by the use of Prot-A conjugated with horse radish peroxidase (HRP). It is known that Prot-A is mainly reactive against human IgG and not IgM antibodies.

4.4. Evaluation of IgG and IgM antibodies titers in the collected sera

All tested sera were analyzed for their reactivity against both S and M COVID-19 expressed proteins, and both IgG and IgM were evaluated. Results of the current study showed that all the tested samples against both S and M proteins were negative for the presence of IgM

Table 6 Summary for the IgG antibody titers of the tested sera samples against S and M proteins used in ELISA test.

	Sera with single vaccination dose		Sera with two vaccination doses		Sera with three vaccination doses	
Ab Titer	S protein	M protein	S protein	M protein	S protein	M protein
1:100	4	12	0	8	0	5
1:200	14	4	7	21	0	8
1:400	0	0	28	7	23	10
Negative	6	8	5	5	3	3

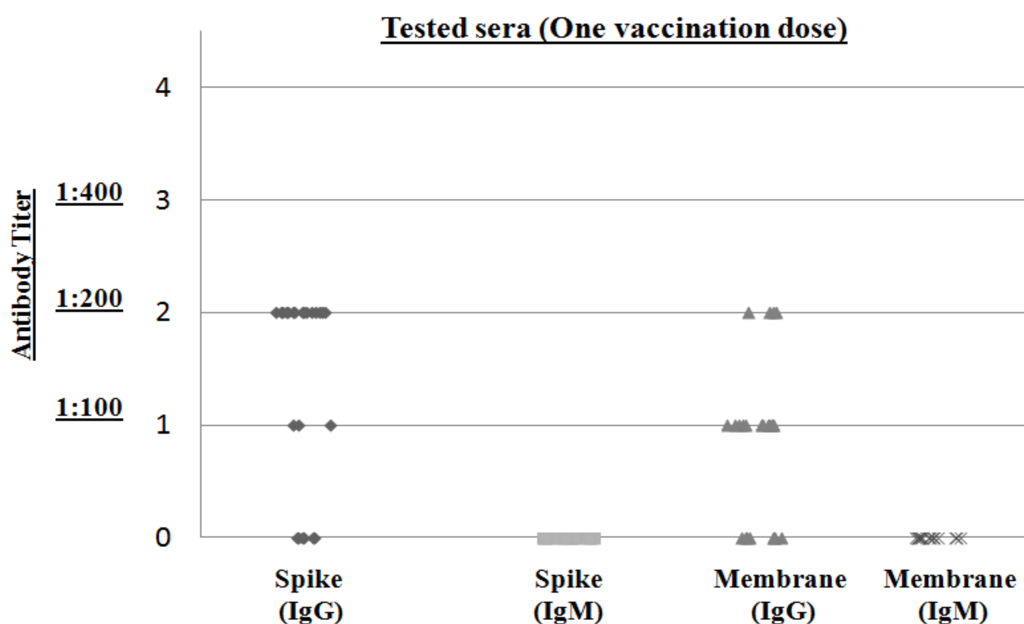

Figure 7 Dot representation of IgG Antibody titer from sera of one dose of vaccinated individuals against S and M proteins, with a total of 41 tested samples

antobodies. So the below results are only showing the obtained ELISA results of IgG antibody titer against COVID-19 S and M proteins. The obtained ELISA results of IgG antibody titer against COVID-19 S and M proteins were divided into three groups according to the number of vaccine doses. Figure 7 and Table 6 display the ELISA

findings that indicate the responsiveness of individuals' sera who have only had one dose of a vaccine three years ago. Generally speaking, studied sera's reactivity to the S protein was higher than their reactivity to the M protein. This is true for all tested serum samples from various vaccination dosage groups as well. Six of the twenty-four samples that were examined reported negative, indicating they had no reactivity to either the S or M proteins. With an antibody titer of 1:100 and 1:200, respectively, 4 and 14 of the remaining examined samples demonstrated reactivity with S protein. The findings for the M protein were 12 with an antibody titer of 1:100 and 4 with a 1:200 antibody titer (Figure 8, Table 6). The IgG antibody titer in none of the samples was 1:400.

When the S protein was used in the ELISA test, the results for the examined serum samples, which represent individuals who received two doses of vaccine, were 7 and 28, with antibody titers of 1:200 and 1:400, respectively. When the M protein was used, the results were 8, 21, and 7, with antibody titers of 1:100, 1:200, and 1:400, respectively. Five samples were negative for both S and M proteins (Figure 9, Table 6). Based on this initial analysis it was anticipated that the individuals whom have received three doses of vaccinations showed a higher IgG antibody titers although still there were three sera samples that didn't showed a reactivity to either S or M proteins. The number of samples that showed reactivity with IgG in an antibody titers of 1:400 was 23 out of the 26 tested sera once using the S protein antigen, compared to 10 samples with an antibody titer of 1:400 with the same sera were tested against M protein (Figure 9, Table 6). 5 and 8 of the analyzed sera samples against the M protein showed an antibody titer of 1:100 and 1:200 respectively.

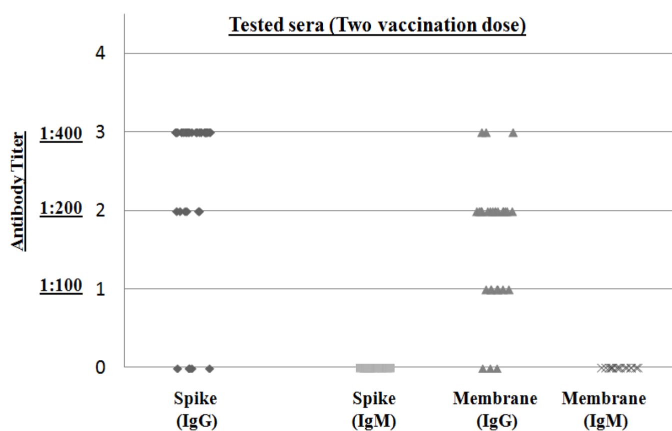

Figure 8 Dot representation of IgG Antibody titer from sera of two doses of vaccinated individuals against S and M proteins, with a total of 26 tested samples.

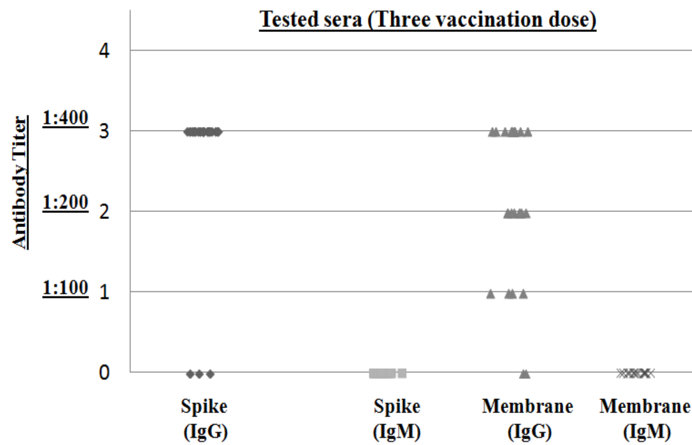

Figure 9 Dot representation of IgG Antibody titer from sera of three doses of vaccinated individuals against S and M proteins, with a total of 26 tested samples.

Table 7-A: Information of the collected sample sera that were analyzed, together with the associated IgG antibody titers against the S and M proteins utilized in the ELISA experiments.

No	Age	Sex	Vaccine	Doses	Date of vac	Titer (S Antigen)	Titer (M Antigen)
1	29	F	Sputnik	1	2021	1:200	1:100
2	50	M	Pfizer	1	2021	1:200	1:100
3	32	F	Pfizer	1	2020	Negative	Negative
4	32	F	Pfizer	1	2020	Negative	Negative
5	40	F	Sputnik	1	2020	1:100	1:100
6	33	F	Sputnik	1	2020	1:200	1:100
7	35	M	Sputnik	1	2020	1:200	1:200
8	82	M	Pfizer	1	2021	Negative	Negative
9	34	F	Pfizer	1	2021	1:200	1:200
10	42	M	Sputnik	1	2021	1:100	1:100
11	39	M	Sputnik	1	2021	Negative	Negative

Table 7-B

12	39	M	Sputnik	1	2021	1:200	1:100
13	33	F	Pfizer	1	2020	S1:100	1:100
14	44	F	Sputnik	1	2020	Negative	Negative
15	29	F	Sputnik	1	2020	1:200	1:200
16	31	M	Sputnik	1	2020	1:200	1:200
17	80	M	Pfizer	1	2021	Negative	Negative
18	42	F	Pfizer	1	2021	1:100	1:100
19	40	M	Sputnik	1	2021	1:200	1:100
20	34	M	Sputnik	1	2021	1:200	1:100
21	33	M	Pfizer	1	2021	1:200	1:100
22	37	M	Sputnik	1	2021	1:200	1:100
23	31	M	Pfizer	1	2021	1:200	Negative
24	27	M	Sputnik	1	2021	1:200	Negative
25	31	F	Pfizer	2	2020	1:400	1:200
26	48	M	Pfizer	2	2020	1:200	1:200
27	38	M	Pfizer	2	2020	1:400	1:200
28	44	F	Pfizer	2	2020	1:400	1:200
29	59	M	Pfizer	2	2020	Negative	Negative
30	57	M	Pfizer	2	2020	1:400	1:400
31	47	M	Pfizer	2	2020	1:400	1:200
32	46	M	AstraZeneca	2	2020	1:400	1:200
33	29	M	Pfizer	2	2020	1:400	1:200
34	60	M	Pfizer	2	2020	Negative	Negative
35	49	M	AstraZeneca	2	2020	1:400	1:400
36	41	M	Pfizer	2	2020	1:400	1:100
37	36	M	Pfizer	2	2020	1:200	1:100
38	54	M	Pfizer	2	2020	1:400	1:200
39	45	M	Pfizer	2	2020	1:400	1:100
40	32	M	Pfizer	2	2020	1:400	1:100
41	50	F	Pfizer	2	2020	Negative	Negative

Table 7-C

42	31	M	Pfizer	2	2021	1:200	1:200
43	19	F	Pfizer	2	2021	1:400	1:100
44	40	M	Pfizer	2	2021	1:400	1:200
45	27	F	Pfizer	2	2021	1:200	1:400
46	61	M	Pfizer	2	2021	1:200	1:200
47	55	M	Pfizer	2	2021	1:400	1:200
48	28	F	AstraZeneca	2	2021	1:400	1:100
49	53	M	Pfizer	2	2021	1:400	1:200
50	43	F	AstraZeneca	2	2021	1:400	1:400
51	40	M	Pfizer	2	2021	1:400	1:200
52	47	M	Pfizer	2	2021	1:400	1:200
53	48	F	Pfizer	2	2021	Negative	Negative
54	39	F	AstraZeneca	2	2020	1:400	1:200
55	33	F	Pfizer	2	2020	1:400	1:200
56	34	F	Pfizer	2	2020	1:200	1:100
57	24	F	Pfizer	2	2020	1:400	1:400
58	33	F	Pfizer	2	2020	1:400	1:200
59	45	M	Pfizer	2	2020	1:400	1:100
60	46	M	Pfizer	2	2020	1:200	1:200
61	32	M	Pfizer	2	2020	1:400	1:200
62	47	M	Pfizer	2	2020	Negative	Negative
63	34	M	Pfizer	2	2020	1:400	1:200
64	46	F	Pfizer	2	2021	1:400	1:200
65	34	F	Pfizer	2	2020	1:400	1:400
66	59	F	Pfizer	3	2021	1:400	1:400
67	49	F	Pfizer	3	2020	1:400	1:200
68	41	M	Pfizer	3	2020	1:400	1:400
69	37	M	Pfizer	3	2020	1:400	1:400
70	51	M	Pfizer	3	2020	1:400	1:200

Table 7-D

71	41	M	Pfizer	3	2020	1:400	1:400
72	38	M	Pfizer	3	2020	1:400	1:200
73	34	M	Pfizer	3	2020	1:400	1:100
74	37	M	Pfizer	3	2020	1:400	1:200
75	55	M	Pfizer	3	2021	1:400	1:400
76	37	ذكر	Pfizer	3	2021	1:400	1:200
77	55	M	Pfizer	3	2021	1:400	1:400
78	38	M	Pfizer	3	2021	Negative	Negative
79	25	M	Pfizer	3	2021	1:400	1:200
80	41	M	Pfizer	3	2021	1:400	1:100
81	31	M	AstraZeneca	3	2021	1:400	1:400
82	62	M	Pfizer	3	2021	Negative	Negative
83	56	M	Pfizer	3	2022	1:400	1:200
84	56	M	Pfizer	3	2022	1:400	1:100
85	46	M	Pfizer	3	2020	1:400	1:400
86	35	M	Pfizer	3	2020	1:400	1:200
87	44	F	Pfizer	3	2021	1:400	1:400
88	31	F	Pfizer	3	2020	1:400	1:400
89	56	F	Pfizer	3	2021	1:400	1:100
90	52	F	Pfizer	3	2020	1:400	1:400
91	60	M	Pfizer	3	2021	Negative	Negative
92	30	F	Normal	Norma	Normal	Negative	Negative
93	50	M	Normal	Normal	Normal	Negative	Negative
94	37	F	Normal	Normal	Normal	Negative	Negative
95	50	F	Normal	Normal	Normal	Negative	Negative
96	50	M	Normal	Normal	Normal	Negative	Negative
97	19	M	Normal	Normal	Normal	Negative	Negative
98	56	F	Normal	Normal	Normal	Negative	Negative
99	14	M	Normal	Normal	Normal	1:100	Negative
100	45	F	Normal	Normal	Normal	Negative	Negative
101	37	F	Normal	Normal	Normal	Negative	Negative

Table 7-E

102	59	F	Normal	Normal	Normal	Negative	Negative
103	21	M	Normal	Normal	Normal	Negative	Negative
104	50	F	Normal	Normal	Normal	Negative	Negative
105	20	M	Normal	Normal	Normal	1:100	Negative
106	51	F	Normal	Normal	Normal	Negative	Negative
107	45	F	Normal	Normal	Normal	Negative	Negative
108	55	F	Normal	Normal	Normal	Negative	Negative
109	28	M	Normal	Normal	Normal	Negative	Negative
110	33	F	Normal	Normal	Normal	Negative	Negative

Chapter Five

Discussion

The current study was implemented to evaluate an SARS CoV2 immunological antibodies based on testing SARS CoV2 IgG and IgM antibodies qualitatively and quantitively. In addition, to provide a continuous source of SARS CoV2 surface protein antigenic material in sufficient quantities by using cloning technique to be utilized in indirect ELISA tests.

S protein is the most significant viral structural protein in terms of SARS CoV2 pathogenesis because it promotes receptor binding and virus–cell membrane fusion and is rich in antigenic and neutralizing epitopes, so it was the most suitable antigen for such test (Dong, 2021). This research was also applied because of a general health care about COVID-19 as the disease has been reported in almost all over the world.

Spike S proteins are able to identify Angiotensin converting enzyme 2 (ACE2) upon an outer layer of endothelial cells. Additionally, spike S proteins can detect the presence of the angiotensin converting enzyme 2 (ACE2) on the outer layer of endothelial cells, as well as any cell that may be infected with SARS-CoV2.

We obtained 91 blood serum samples from hospital patients in Palestine over the course of our inquiry. Using an ELISA approach antibody screening, we were able to

determine that the maximum occurrence of SARS-CoV2 in a community was indicated by a positive rate of 70.09 percent, even if the number of specimens may have been inadequate. The results of our investigation showed that the total number of SARS CoV2 cases and incidence was still significantly higher among those who had not received vaccine than among those who had; additionally, the COVID-19 virus was more common among those who had received fewer vaccine doses than among those who had received more. To further assess the results of this study, large number of sample will be needed for future antibody-screening studies. The findings of the ELISA analysis showed that a number of the previously affected people tested negative.

This may be the consequence of a decrease within antibody titer brought on by successful treatment, a prolonged period of time (above five month) between illness and serum sample collection, or the fact that those people did not obtain a booster injection shots of vaccination following infection, all of which could have detrimental effects. It's probable that the same explanation applied to some of the specimens be negative, even though they had gotten the vaccination.
The ELISA technology method was used to determine the immunity by measuring the IgG and IgM antibody titers of the various tested serum samples against SARS CoV2 crude antigen, these samples were gathered from present vaccinated patients or previous infected patients and normal not vaccinated individuals, and the test results had been reported to demonstrate high sensitivity & specificity of the ELISA technology in antibodies detection, in addition high sensitivity & specificity once compared to other techniques recommended serological methods like western blot and chemiluminescence method as well.

Merely keeping an eye on and evaluating findings on vaccination efficacy has demonstrated that the likelihood of an outbreak disease increases with a reduction for neutralizing antibodies. In the meanwhile, various indicators are used by experts in order to determine if immunizations are effective. This includes evaluating immunization efficacy in an actual life-settings, during a period of time as well as among particular groups. (Krammer, 2021)
The Israeli government decided to use this method for releasing SARS-CoV2 boosters during a summertime in the year of 2021. Data across the nation showed that those who received their vaccinations early in the calendar year had a higher likelihood of developing significant acute infections compared to individuals who received their

shots later. Since there currently is no correlation between COVID-19 prevention and vaccination, you cannot utilize an antibody testing to determine your level of protection versus a coronavirus following an unexpected infection or immunization. (Krammer, 2021)
The indirect ELISA method has a higher sensitivity than the direct ELISA method because it uses fewer labelled secondary antibodies to bind the main antibody. It is also less expensive than the direct ELISA approach since it uses less labelled antibodies (Bulter, 2000)

The analysis was conducted to get results about the Corona virus, its spreading and its effect on people, and then enter this data as input into the laboratory database, and this study show that there is a clear difference between the ELISA results of vaccinated and unvaccinated healthy people. The vaccinated individuals show higher titer than unvaccinated normal individuals, that means presence of high number of antibodies against SARS-Cov2 in their blood.

We recommend as well to test specificity & sensitivity applying ELISA test against other types of Corona viruses variants to be stronger in validation issue.

Future work

It's recommended for future to research about how corona disease impact on the immune system by using other several techniques and addition new developed tests to do validation. Can making further research about other antigens rather than S and M. need to be further analysis via antibody-screening investigations using larger number of tested individuals' specimens. For future researches also recommended to verify the specificity of this ELISA test applying the used S and M antigens, and this can be done by using other kinds of Corona viruses or other respiratory viral infections. On other hand, there are many animal viruses can infect human to implementing further research about, moreover the COVID 19 is have a high capacity in invading animal cells its open door to do many future researches around this field. Future studies could be done to understand the way animal viruses pass throw species barriers then successfully infect humans it will empower a mitigation of future zoonotic incidents. Characterization an antibody reaction for vaccination is vital to devise strategies to future vaccines.

Chapter Six

Conclusions

In this study, the IgG and IgM antibodies titers against SARS-CoV-2 were evaluated and compared among vaccinated and non-vaccinated individuals in Palestine. For antibody titers and serotypes measurement in serum ELISA technology performed. Evaluation by targeting SARS-CoV-2 antigens especially (S) antigen & (M) antigen as capture antigens by antibodies. Better understanding of the immune system and molecular biology of coronaviruses is critical to elucidate their emergence, origin, evolution, diversity, pathogenesis, epidemiology and techniques deal with them as ELISA method which is sensitive and specific in identifying corona epitopes.

Complete information of molecular characteristics of circulating coronaviruses is important for development of effective diagnostic tools to detect these viruses. Simply the antigens S and M that shows a high reactivity in ELISA test represent the most of positive serum molecules which give us a good interpretation for the prevalence reactive antibodies against corona virus for long period time, among vaccinated patients in Palestine.

This thesis aims to evaluate of immune system via examination by assessment the antibodies titer of people infected with the COVID19 disease. The research included a sample of eighty-five people who had received vaccination or were previously infected with the Corona virus, where the immunological results of the people were analyzed. Recording the results of laboratory analyzes and saving them in a database using a computer. An analysis was conducted in order to obtain results about the Corona virus, its spread and its effect on people, and then enter this data as input into the laboratory database, and it was found from this study that there is a clear difference between the data of vaccinated and healthy people, as it appeared that the vaccinated show higher titer than unvaccinated normal individuals.

This study demonstrates the importance of knowing the impact of the Corona virus on people and how to find solutions to control and limit the transmission of the COVID 19 in order to find an appropriate treatment for this virus and improve new vaccination.

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Abstract in Arabic

تقييم عيارات الأجسام المضادة والأنماط المصلية لدى الناس الذين تم تطعيمهم ضد كوفيد-19

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الملخص

سارس كوفيد 19 هو فيروس كورونا جديد ظهر في عام 2019، ويصنف الآن **المقدمة:**

-SARS يعتبر SARS-CoV. ضمن جنس الفيروسات التاجية، مرتبطًا ارتباطًا وثيقًا ب

شديد الإمراض لدى البشر، ويصنف على أنه ممرض من المستوى الثالث للسلامة CoV-2

أدت إلى وفاة أمرًا صعبًا نسبيًا بسبب طبيعته المعدية التي البيولوجية، مما يجعل التلاعب به

.عدد كبير من الأشخاص

استجابة الجهاز المناعي وقياس عيار الأجسام المضادة، تم جمع عينات مصلية **الطريقة:** لتقييم

أو كانوا COVID-19 من 85 مريضا في مستشفى بيت جالا الذين تم تطعيمهم ضد مرض

الأجسام المضادة، تم مرضى مصابين سابقا ومن متبرعين سلبيين غير مطعمين. لتقييم عيارات

في المختبرات، وتقييم عدوى الفيروس المناعي المرتبط بالإنزيمELISAإجراء تكنولوجيا

وتقييمها وتحديدها للكشف عن عيارات2 -SARS-CoV ل SPIKE S وMوبروتينات

في مصل المرضى الذين تم SPIKEالمرتبطة بال للفيروس الأجسام المضادة المعادية

. وقياس ELISAفي المستشفى باستخدام الانزيم التجاري وتقنية Covid19 تطعيمهم ضد

علاوة على ذلك، تم . BSLفي ظل ظروف SARS-CoV-2 الأجسام المضادة المعادية ل

تحت ظروف مستوى SARS-CoV-2 للأجسام المضادة المعادية ل ELISA تطوير فحص

تسجيل نتائج التحاليل المخبرية وحفظها في قاعدة بيانات باستخدام تم.السلامة البيولوجية

الحاسوب.

النتيجة: تم إجراء تحليل وتقييم لعيارات الأجسام المضادة، والحصول على نتائج حول فيروس كورونا وانتشاره وتأثيره على الناس. حيث تم تحليل النتائج المناعية والميكروبيولوجية للأشخاص، ومن ثم إدخال هذه البيانات كمدخلات في قاعدة البيانات المخبرية ، وتبين من هذه الدراسة أن هناك وجود تباين واضح بين بيانات الأشخاص المطعمين والأشخاص الأصحاء غير الملقحين، حيث أظهرت النتائج أن المطعمين لديهم عيارات أعلى من الأفراد الطبيعيين غير المطعمين.

وفي الختام هدفت هذه الدراسة إلى تقييم فعالية لقاح COVID-19 على المدى الطويل لسكان فلسطين من خلال تحليل استجابتهم للقاح بعد عامين، بناء على مستويات عيار الأجسام المضادة ل SARS-CoV-2 بروتين S و M، وتوضح هذه الدراسة أهمية معرفة تأثير فيروس كورونا على الناس، وفعالية اللقاحات، ووجود الأجسام المضادة المعادية COVID-19 لتقييم نظام المناعة من خلال فحص عيار الأجسام المضادة والأنماط المصلية، والتي يمكن تنفيذها وأدائها بسهولة داخل مختبر مستوى 3 للسلامة البيولوجية (BSL) لتقييم حدوث الأجسام المعادية ضد SARS-CoV-2 وتقييم كل من المستضدات S و M والأنماط المصلية للسيطرة والحد من انتقال COVID-19 من أجل إيجاد علاج مناسب لهذا الفيروس وتحسين التطعيم الجديد في فلسطين.

القدس – فلسطين

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