Deanship of Graduate Studies

Al-Quds University

"Evaluation of Western blot analysis for the detection of COVID-19 infected and vaccinated serum samples against different viral specific antigen preparations"

Aseel Basem Sami Eqneiby

M.Sc. Thesis

Jerusalem – Palestine

1444– 2023

" Evaluation of Western blot analysis for the detection of COVID-19 infected and vaccinated serum samples against different viral specific antigen preparations "

Student Name: Aseel Basem Sami Eqneiby

B.Sc: Hebron university Country: Hebron /Palestine

Supervised by: Dr. Rasmi Abu-Helu

Co-supervisor: Dr. Ibrahim Abbasi

A thesis submitted in partial fulfillment of Requirement for the degree of Master of Medical Laboratory Science / Diagnostic Microbiology and Immunology Track/ Faculty of Health Professions/ Al-Quds University

1444 – 2023

Al-Quds University
Deanship of Graduate Studies
Faculty of Health Profession

Thesis Approval

" Evaluation of Western blot analysis for the detection of COVID-19 infected and vaccinated serum samples against different viral specific antigen preparations "

Prepared By: Aseel Basem Sami Eqneiby
Registration number: 21911801

Supervisor: Dr. Rasmi abu-Helu
Co-Supervisor: Dr. Ibrahim Abbasi
Master thesis submitted and accepted. Date:14/1/2023

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

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

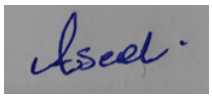
Jerusalem – Palestine

1444 – 2023

Declaration

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:



Name:Aseel Basem Sami Eqneiby

Date:14/1/2023

Dedication

I dedicate this work to my Lovely Parents

I dedicate this work to my dear husband salame

I dedicate this work to my doctors

I dedicate this work to my sisters and my brothers

I dedicate my work to all teachers who teach me

I dedicate my work to my friends

I dedicate my work to everyone who believed me.

Acknowledgment

بسم الله الرحمن الرحيم

First, and most of all, I would like to thank God on what he granted me. I thank him for aspiring me to continue through master's degree in Medical Laboratory Science.

I would like to thank who facilitated my path and encouraged me to continue educational process (my lovely parents and my dear husband Salame Muhtaseb).

I would like to give a special thanks to my supervisor, Dr Rasmi Abu. Helu and Co-Supervisor, Dr. Ibrahim Abbasi, for their support, directing, suggestions and patience throughout the process of guidance. It was a pleasure working with them.

I would like to thank my college faculty of medical laboratory science department; Al-Quds University, the whole teachers, doctors and technicians.

Finally, I would like to thank my friend Tamer Shabana and everyone who helped and supported me to continue this thesis.

Signature:

Abstract:

Background: Sever acute respiratory syndrome coronaviruses 2 (SARS-Cov-2) are member of the subfamily coronaviridia that cause corona-virus disease 2019 (COVID-19). Coronaviruses are a positive-sense single-stranded RNA (+ssRNA) non-segmented genome of 30–32 kb with a special capsid and spikes protein. The main target of COVID-19 virus is ACE2 that is located in the lung tissues. The main objective of this study is to evaluate different antigenic preparations in Western-blot analysis against different status of serum samples (vaccinated infected and non-vaccinated infected sera). In this test,IgG and IgM antibodies were evaluated.

Method: New M13 phage display plaques were identified that contain 12 amino acids epitopic regions were selected against pooled positive COVID-19 sera. Two groups of epitopic 12 amino acids were used together with spike and membrane recombinant proteins in the current evaluated Western-blot analysis. The used sera in this study were of three types: (20 COVID-19 positive and vaccinated serum samples, 10 positive non-vaccinated serum samples, and some negative serum samples).

Results and conclusion: In general the use of M13 phage display plaques containing 12 amino acids that were selected using COVID-19 pooled infected sera; were proved to be more reactive with vaccinated and even infected and non-vaccinated COVID-19 serum samples, this compared to the use of spike or membrane recombinant protein antigenic preparations. It was clearly seen that these antigenic preparations were only immunoreactive with IgG and not IgM, which suggests their usefulness in detecting cases with high IgG antibody titers and never in acute COVID-19 infections. This finding could emphasis the potential of using the M13 phage display plaques containing 12 amino acids in evaluating vaccinations procedures against COVID-19.

Table of contents

List of Figures:

Abbreviation:

ACE2	Angiotensin-Converting Enzyme 2
APCs	Antigen presenting cells
COVID-19	Corona virus disease 2019
DNA	deoxyribonucleic acid
ELISA	Enzyme-Linked-Immunosorbent Assay
E. coli	Escherichia coli
FCS	Fetal Calf Serum
HRP	Horseradish peroxidase
IPTG	Isopropyl-beta-D-thiogalactoside
MERS	Middle East Respiratory Syndrome
MHV	Mouse Hepatitis Virus
MHC	major histocompatibility complex
NC	Nitrocellulose Membrane
ORF	Open reading frame
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffer Saline-Tween 20
PCR	Polymerase Chain Reaction
RT-PCR	Real time polymerase chain reaction
RNA	ribonucleic acid
SARS CoV2	Sever acute respiratory syndrome Coronavirus 2
S protein	Spike protein
SDS-PAGE	Sodium-Dodecyl sulfate Polyacrylamide Gel Electrophoresis
WHO	world health and organization
Nsps	Non- structural protein
DDP4	Dipeptidyl peptidase
CV	Covalence plasma
ARDS	Acute respiratory distress syndrome

Introduction:

Severe acute respiratory syndrome coronaviruses 2 (SARS -COV-2) are member of the subfamily coronaviridia ,and a novel viruses that causes coronaviruses disease 2019 (COVID-19) (Wanyin Tao, 2021),metagenomics analysis and genomic sequencing study of COVID-19 revealed that this viruses was identical 79.6% to the genetic sequence of SARS COV-1 (SARS outbreak in 2002) leading world health and organization(WHO) to call it as a novel COVID-19 (Umakanthan *et al.*, 2020).

At the beginning of the COVD-19 outbreak that found an association between the origin of COVID-19 and animal market in Wuhan city where live animal are sold but after the progression of the COVID-19 human to human transmission become the most important way of spreading the virus all over the world (vicirajmet etal.2020).

Aerosols and respiratory droplets both are important routes for transmission of COVID-19 (*benjamins et al.2020)* , Because of the rapid and uncontrolled spreading of the virus and increase in the number of injuries throughout the world the WHO identified the COVID-19 as a pandemic(Okba *et al.*, 2020) .

Many studies indicated that the nasal epithelium in particular is a first site where the SARA-Cov-2 infection and replication, which lead to disturbance in sense of smell and taste, appear mainly in most mild COVID-19 patients (*benjamins et al.2020*). The main target of COVID-19 virus is ACE2 that is located in the lung tissues (*adil et al.2020)* , Several studies indicated that the incubation period is between 2 and 14 days with an average of 5 days (Sapkal *et al.*, 2020).

Coronaviruses typically are enveloped viruses with a positive sense single strand RNA genome with size rang around 26 to 32 kilo bases (Mujeeb et al.2020) , after the emerge of SARS-COV-1 (severe acute respiratory syndrome coronaviruses) and middle east respiratory syndrome coronavirus (MERS-COV) COVID-19 is another member of coronavirus family that has a strong ability to infect human beings (Cui, Li and Shi, 2019) .

Current research shows that COVID-19 has more than 85% homology with SARS-COV -1(*Benjamins et al.2020*) , According to most COVID-19 species the S1-subunit of spike protein (S protein) has a low sequence identity to others including

that of SARS-COV-1 on which it relies in cell binding(Zhiqiang et al.2020), On the other hand the S2- subunit which suggest it relies in the fusion mechanism of the virus to the host cell is highly conserved sequence which has about 90% identity in amino acid sequence with other coronaviruses species including SARS- COV-1 (Zhiqiang et al.2020), whereas according to MERS virus have less homology with COVID-19 this is due to difference in the spike protein genetic variation which lead to make MERS virus have some different in the receptor target which is mainly used dipeptidyl peptidase 4 (DPP4) also name as CD26 as a receptor that is located in non-ciliated bronchial epithelial lung cell(Cui, Li and Shi, 2019) .

In this study **"identification and diagnosis of the SARA-Cov2 viruses via western blot analysis"** we will examine the presence of IgG and IgM antibodies in the infected patient serum by using a highly sensitive immunoassay test based on viruses protein that is called the western blot analysis.

Problem statement:

The current method of Diagnosis of covid19 is based on nucleic acid RNA biological detection by reverse transcriptase polymerase chain reaction (RT-PCR) technique using nasopharyngeal swabs or deep sequencing .These method depends on the detection of presence of the viral genome in sufficient quantities inside the sample that have been collected and it cannot be used to monitoring development of the disease at different stages which made this technique has some limitation in COVID-19 virus detection that is what pulled us to search for another technique which can use it as a confirmatory test for the presence of COVID-19 and determined

seroconversion of this virus with highest sensitivity and specificity at different stage of the disease and lowest consuming time and cost.

The determination of COVID19 is highly dependent on the detection of IgM or IgG antibodies specific to various viral antigen including, but not limited to, the spike glycoprotein (S1 and S2 subunits, binding receptors domain (RBD)) and a nucleocapsid protein so we are going to use the spike protein detection as the most immunogenic and external Ag for COVID-19 via western blot technique.

Objectives and aim of the study:

The main aim of this method is to highlight the possible advantages of serological assays for identification of COVID-19 antibodies evoked against spike patient subunit1 (S1) and other membrane proteins. The tested sera will be collected from COVID-19 patients who are PCR confirmed for their infection. Our assumption is that the detection of S1 antibodies is more specific than detecting total spike protein antibodies targeting S2 domain of the COVID-19 which can cross react with the SARS-COV-1(Zhiqiang et al.2020).

So we will examine the presence of IgG and IgM antibodies inside the infected patient serum by using a highly sensitive immunoassay test based on viruses protein that is called the western blot analysis.

The following are the main specific objectives of the current study:

1- To perform Western blot serological test using COVID-19antigen protein, and identify the type of the evoked antibodies (IgG or IgM).

2- To correlate the DNA based results with the used serological tests among vaccinated and not vaccinated if possible.

Chapter one: Literature review

1.1 History:

Coronaviruses have been noticed as respiratory pathogen that attack the respiratory tract system. The first infection with the coronaviruses was observed in Guangdong Province of china at 2002 and ended at 2003 of year and that spread out all over the world under the name of SARS COV-1(pal et al.2020) . The patients with SARS Cov-1 virus suffered from severe acute upper and lower respiratory syndrome with pneumonia such as cough and dyspnea(Guarner et al.2020).

Years later a newly coronaviruses was emerged in the middle east and causes respiratory inflammation name as MERS-COV(Guarner et al.2020), all injures with MERS have been appeared in people in or near the Arabian Peninsula(Guarner et al.2020), moreover, the fatality rate between the patients suffering from the MERS-COV was higher in comparison to SARS-Cov-1 virus furthermore it still circulating(Guarner et al.2020).

In December 2019 in Wuhan city center a newly emerged viruses was observed which is affected respiratory tract system and causes pneumonia infection [13]. In February 2020 the viruses named as COVID-19 virus where is emerged all over the world and resulted a pandemic infection named as COVID-19 disease(Pal *et al.*2020) COVID-19 was found to infect more human beings than either of its predecessors that include the SARS-CoV-1 and the Middle East respiratory syndrome virus (MERS)(Guarner, 2020).

COVID-19 viruses emerged in Wuhan city center and turned into a global disease(Salian *et al.*, 2021). COVID-19 is recommended as a zoonotic disease that transmits via direct contact with body fluid of infected animals or via areolas and droplets of infected people(Salian *et al.*, 2021). Very increased rate of injurers with the COVID-19 in very short time drew global attention to this virus (Xu *et al.*, 2020). The corona virus which has been emerged in 2019 was renamed as COVID-19 due

to it is highest similarity with the severe acute respiratory tract infection SARS – COV-1(Xu *et al.*, 2020).

1.2 Biology:

1.2.1 COVID-19 Structure :

Coronaviruses are a positive-sense single-stranded RNA (+ssRNA) non-segmented genome of 30–32 kb with a special capsid and spikes protein that surrounded it(de Wilde *et al.*, 2018), COVID-19 has a specific structure protein on his surface that play an important role in virus pathogenesis and complication(Pal *et al.*2020) the coronavirus genome arranged in two region 5'end region that contains open reading frames which encoded genome protein responsible in viral replication and 3 ' region that contains the structural proteins arranged in the order hemagglutinin esterase (HE), spike (S), small membrane (E), membrane (M) and nucleocapsid (N) and internal (I) protein encoded within the N gen (Weiss and Leibowitz, 2011) .

A group of researchers found that the Corona virus has a strong association with human respiratory receptors (Xu *et al.*2020), (Zhou et al.2020) representative of a group of experiments that show COVID-19 infected cell and tissues that hold on his surface angiotensin-converting enzyme 2 (ACE2) receptors ,This was considered as a strong evidence that theCOVID-19 viruses enter the host Via binding to ACE2 receptor(Zhou *et al.*2020).

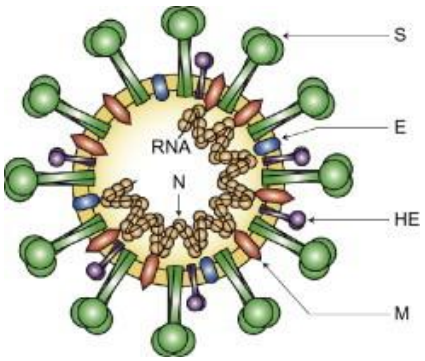


Figure 1: Coronavirus virion structure, which is consist of group of structure protein include of N protein (neucleocapsid protein), HE protein (hemagglutinin-esterase), S

protein (spike), E protein (membrane envelope), M protein (transmembrane proteins)(Weiss and Leibowitz, 2011).

1.2.2 COVID-19 structural proteins:

Spike protein (S): Spike protein is a trimmer protein impeded within the envelop and given the virion the crown-like morphology and appears as a roughly cylinder shape and highly glycosylated and encoded function of receptor binding and fusion of the virus, s protein mediated successful infusion to the host cell by binding to receptor binding domain (RBD) of S1 subunit (Weiss et al .2011)(Rahman *et al.* 2021).

To reach the fusion stage the s protein need to be cleaved by host cell proteases enzyme, many type of protease enzyme included in spike protein cleavage such as trypsin, catharsis, trans membrane protease serine protease-2 (TMPRSS-2), TMPRSS-4 and human airway trypsin-like protease (HAT)(Li *et al.*2005)(Park *et al.*2016)it looks like a lock and key mechanism.

Nucelocapsid protein (N): Nucleocapsid protein attach with RNA genome and form a helical capsid structure found within the viral envelope(Weiss and Leibowitz, 2011), the N protein genome is coated by lipid enveloped and the lipid enveloped is surrounded with protein enveloped due to it is inability of viruses to make their Own lipid they use the host lipid for replication and also play an important role in morphogenesis of the viruses during its life cycle (Rahman *et al.*2021).

Enveloped protein (E): Enveloped protein is transmembrane proteins involved in virion assembly(Weisset et al . 2011), and it plays an important role at the end of the viral life cycle (Rahman *et al.*2021).

Membrane Protein (M): Like an enveloped protein, M protein is a transmembrane protein involved in assembly of viruses(Rahman *et al.*2021).

1.2.3 COVID-19 non-structural proteins:

In addition of the structural protein, the COVID-19 genome expressed about 16 non-structural protein s (NSPs) from NSP(16) called replicas protein which mediated

essential function such as RNA dependent RNA polymerases and some of them act as methylated cap structure on 5' end and other proteins act as precursor protein .Some of replicas protein provide non-essential function in virus- host interaction(Weiss et al.2011).

1.2.4 Spike protein:

COVID-19 genome are made up of 5'and 3 terminal, 5' terminal considered as a major part of the genome that contains open reading frames which encode protein that needed for viral replication 3 terminal contains the five structural proteins included spike protein (S), membrane protein (M),nucleo capsid protein (N), envelope protein (E), and finally hemagglutinin-esterase (HE) protein (Cui, Li and Shi, 2019) . Among these virus virulence antigens, the spike (S) and nucleocapsid protein are the main immunogenic covid19 protein (Okba *et al.*2020). Spike protein gives the characteristic appearance to the virus and the copy number of S protein is 10—times higher than influenza virus and is comparable to HIV(Umakanthan *et al.* 2020).

COVID-19 virus express a huge (approximately 140 KD) glycoprotein name as spike protein(Amanat *et al.*2020) , spike protein are a trimeric protein consist of three intervened chain with identical amino acid sequence each called a protomar(Rahman *et al.*2021).

The spike protein mediated attachment and fusion between viruses and host cell membrane and also between infected an neipharing un-infected cell also it is a major inducer for neutralizing antibodies in a vaccine, Human to human and cross species transmission of COVID-19 mainly depend on spike protein receptor binding domain(RBD) and it is host receptor angiotensin converting enzyme (ACE2) (Cui et al . 2019), different variant of COVID-19 where is isolated from multiple host vary in their receptor binding domain affinities for human ACE2 receptor therefore effect of the virulence of the disease(Cui et al .2019), ACE2 receptor assist viral entry and replication in the host (Cui et al .2019). This receptor expression in high level in the lung tissues which made them the primary tissues target of the COVID-19 virus.

Spike protein that is also called structural proteins are play an important role in assembly and infection of COVID-19 virus, which included S1 and S2 subunits ,S1 subunit contains the receptor binding domain (RBD) (Umakanthan *et al.* 2020) .That is present on the tip of S1 head (Cui et al.2019) ,and it is binds to ACE2 receptor and assist in cells entering . Furthermore, the S2 subunit which facilitates the fusion and entry process (Umakanthan *et al.* 2020) as many researchers reported there is a role of Angiotensin-Converting Enzyme-2 Receptor in transmission of COVID-19,previously the scientific demonstrated that the main role of ACE-2 receptor in the entry and replication of SARS-COV viral genome ,as in the SARA- COV the ACE-2 receptor serve as the entry point of COVID-19 viruses via interaction resulted to recognized between the S protein which located on the surface of COVID-19 and specific receptor that are located on the surface of the host tissue called ACE-2 (angiotensin converting enzyme 2) receptor(Salian *et al.* 2021).

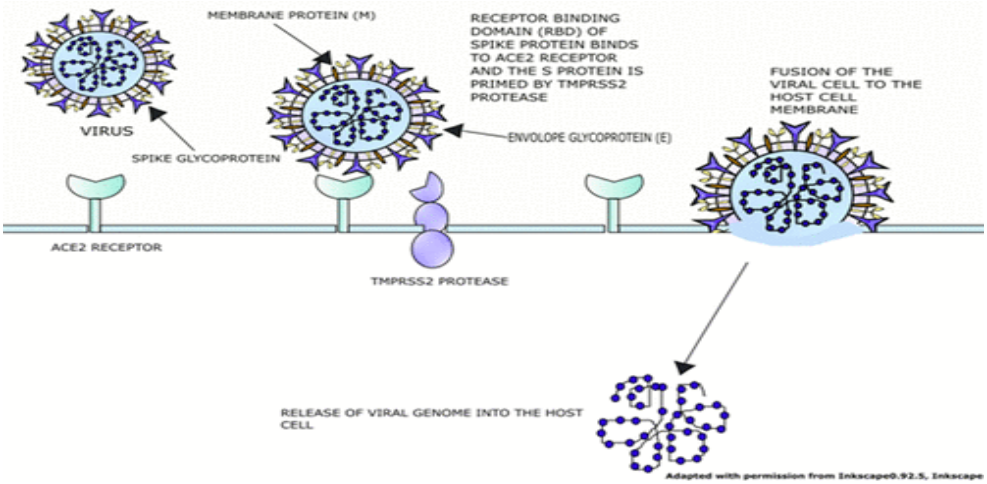


Figure 2. Host receptor interaction with the SARS-CoV-2 spike protein and subsequent viral cell fusion with the host cell membrane(Salian *et al.*2021)**.**

1.3 Life cycle and pathogenicity of COVID-19:

COVID-19 is a zoonotic disease primarily is transmission from infected animal, the first infection are discovered with this infection in the bats in Wuhan city market(Rahman *et al.*2021). Infection is mainly occurring when contact directly or indirectly of mucus membrane (eyes, nose, mouth) with infectious agent particles or droplet (Lau et al .2004). Human to human transmission also has been recorded in high rate that was exhibit highly potential for pandemic of the COVID-19 virus (Pal *et al.*2020). Also there is high possibility of (fecal- oral) transmission of COVID-19 especially in poor sanitation region which facilitate the pandemic of the virus (Geller et al .2012).

As SARS-COV|-1, COVID-19 uses ACE2(Angiotensin converting enzyme 2) as a receptor of the viruses an mainly spreading throw-out the respiratory tract that was made them to categorized it as a respiratory infectious virus (Rahman *et al.* 2021). The attachment of the spike protein(S protein) of COVID-12 virus with it is receptor on the surface of host tissue considered as primarily initiated the binding of the viruses each virus strain has vary on the site of the receptor binding domain (RBD), some of them has a RBD on the N-terminal of S1 like in mouse hepatitis virus (MHV) and the others SARS- COV has the RBD in the C-terminal of S1 subunit(Weiss and Leibowitz 2011)(Pal *et al.*2020) .

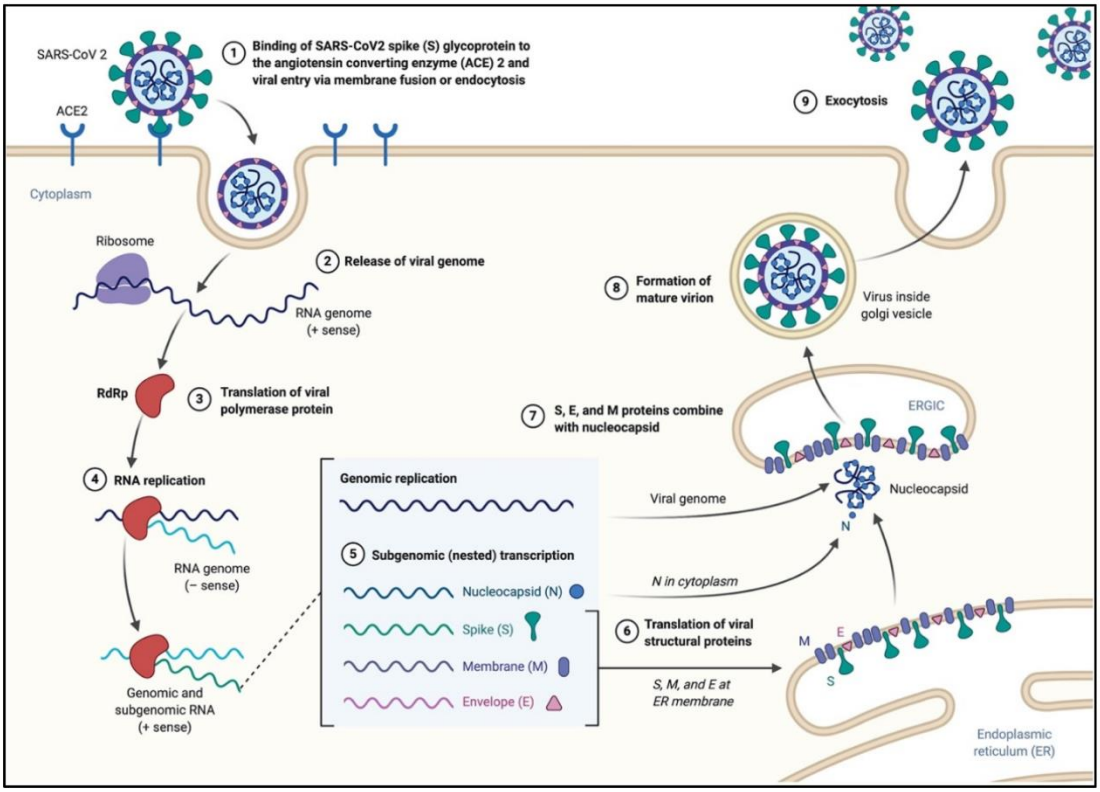


Figure3: This chart describe life cycle of COVID-19 virus , from binding of virus to the host cell with ACE2 receptor and entry of virus via endocytosis to the cytoplasm of host infected cell until the virus excite from the cell through exocytosis(Alanagreh et al .2020).

1.4 COVID-19 and other Betacoronaveridia members:

Corona viruses are a very huge family that infect many types of animal species and human over the past years, in most cases corona viruses infected people and causes in common symptom in most patient Represented in upper respiratory tract infection in general and Bronchial infections in particular as a common cold(Rahman *et al.*, 2021).

Coronavirinae subfamily divided into four major genera alpha (α), beta (β), gamma (γ), and delta (δ) included common type of variant that infect people all over the world such as 229E, NL63, OC43, and HKU1 ,between these variant 229E and NL63 are α-coronaviruses, and OC43 and HKU1 are β-coronaviruses(Rahman *et al.* 2021).Alpha coronavirus and Beta coronavirus infect animal and human whereas Gamma coronavirus, and Delta coronavirus infect birds(Weiss and Leibowitz. 2011)(Li, 2016)(Schwartz and Graham. 2020)(Pal *et al.*2020).

The Covid19 (COVs) belong to:

Tabel1: COVID-19 classification.

genus	Coronavirus(β-coronaviruses)
family	Coronaviridae
order	Nidovirales
Shape	Crown shape
size	80 and 160 nm in length

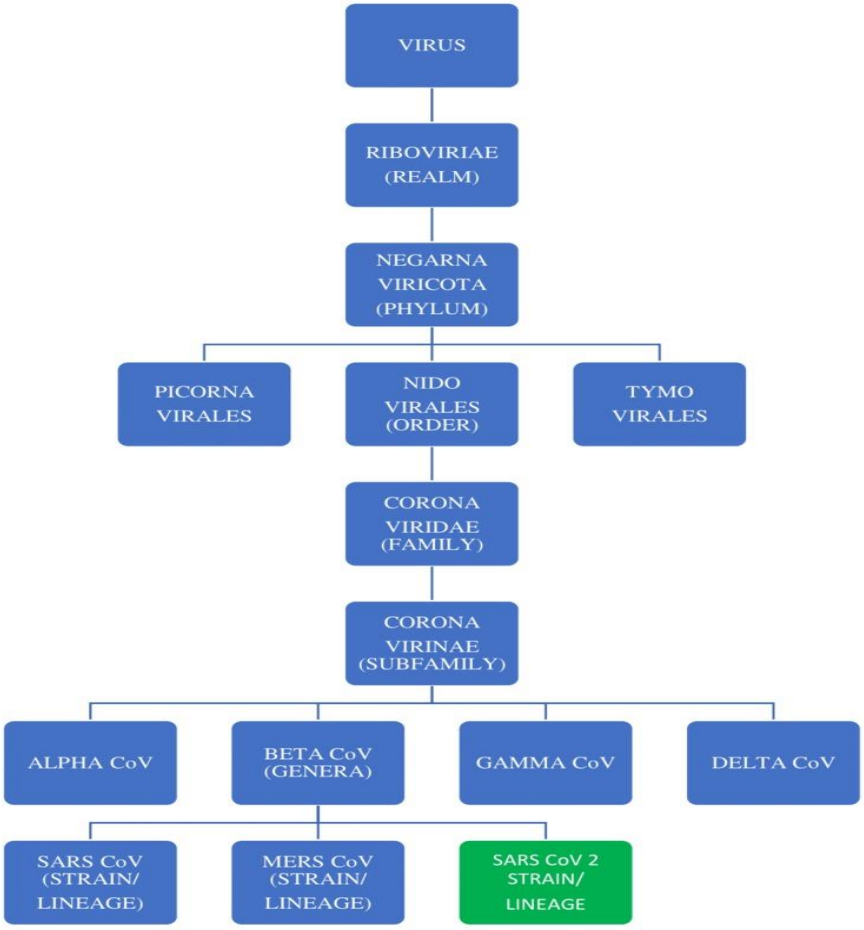


Figure5:The classification of the RNA group of viruses and the origin of SARS CoV-2 COV: coronavirus; SARS COV 2: severe acute respiratory syndrome coronavirus 2; MERS COV, Middle East respiratory syndrome coronavirus; RNA, ribonucleic acid(Pal *et al.*2020)

1.5 Similarities and Differences between SARS and SARS-Cov-2 (COVID-19):

As a SARS-COV-1 , COVID 19 is pulmonary viruses that attacked lung tissues and causes damage in the respiratory tract system ,both of them (Covid19 and SARS) have primarily transmission of the viruses via droplet and areolas of the infected mucus membrane (Salian *et al.*2021) , also both of SARS-COV-1 and COVID 19 have the same shelf half-life on the different surfaces(van Doremalen *et al.* 2020), the incubation time for each one are between 4-7 days and the maximum incubation time is 14 days(Peiris *et al.*2003).

SARS-COV-1 virus has a mortality rate higher than the COVID-19 , and it was founded that there is no mild infection in the SARS-COV-1 unlike in Covid19 viruses which is has mild infection symptom in group of patient(Peiris *et al.*2003). According to World Health Organization (WHO) that indicate of Covid19 an average number of secondary infection by infected patient they have a higher rate of patients with SARS-COV-1(Petrosillo *et al.*2020), all of these characteristic of COVID 19 made it spreading rapidly across all over the world (Salian *et al.*2021)

	SARS	COVID-19
Pre- Transmissibility	NO	YES
Mild Case Transmissibility	NO	YES
Reproduction Number (R_0)	1.7-1.9 (WHO)	2.0-2.5 (WHO); 5.7 with 95% CI: 3.8-8.9 (CDC)
Number of Reported Cases	More than 8000	31.44 million through September 22, 2020
Number of Reported Deaths	774	967,197 through September 22, 2020
Mortality Rate	About 9%	3.1%
Primary Mode of Transmission	Infectious respiratory droplets dispersed from mucous membranes	
Ability to Survive on Surfaces	YES	
Median Incubation Period	4-7 days	
Maximum Incubation Period	14 days	
Potential to cause severe respiratory infection	YES	
Potential to infect CNS and brain	YES	

Table2: Similarities and Differences between SARS and COVID-19.

A purple-shaded items indicate similarity; yellow-shaded represent relative levels, and blue-shaded items are unique to COVID-19 (Petrosillo *et al.*2020)

1.6 Immunity against COVID-19 virus:

Until now there is no guaranteed treatment or vaccine against SARS-Cov-2 infection so the ability of the host immune system to defense against the pathogenic infection mainly COVID-19 virus is the best way to act against virus which is gave the host cell natural immunity (Chowdhury *et al.* 2020).

Initially, the innate immune system is activated immediately after the virus exposure to eliminate the pathogen via antigen presentation and macrophages processes of the pathogenic cell mainly COVID-19 virus(Castro Dopico *et al.* 2022), once the COVID-19 virus enter to the host cell lead to inflame activated inside of macrophages and epithelial cell and also in some of endothelial cell to release of the pro-inflammatory molecules such as a cytokines and interleukin mainly IL-1b and IL-18 to mediated the inflammation especially in severe infection of COVID-19 virus(Azkur *et al.*2020).

T-cell activation via MHC class I molecules which are holed on the surface of epithelium which presented antigen virus to $CD8^+$ cytotoxic T cells to destroyed the

virus via apoptosis and activation of the adaptive immune system (Azkur *et al.* 2020)(Castro Dopico *et al.*2022).

T and B cell are activation in response to innate immune cell and antigen presenting cell (APC)(Castro Dopico *et al.*2022), B-cell can be activated directly via the viruses recognition or interact with activated CD4+ T cells(Azkur *et al.* 2020).At the beginning of infection B-cell produced short –lived term IgM antibodies(Abs) followed by class switching to class of Abs(Newell *et al.* 2021)(Castro Dopico *et al.* 2022), more and more Production of highly virus-specific antibodies incorporation with T and B memory cell production (Castro Dopico *et al.* 2022).

in a few several day of exposure to COVID-19 viruses IgM antibodies production and peak within 7 days of infection, antibodies switching IgA and IgG antibodies are production several days after IgM(Azkur *et al.* 2020).

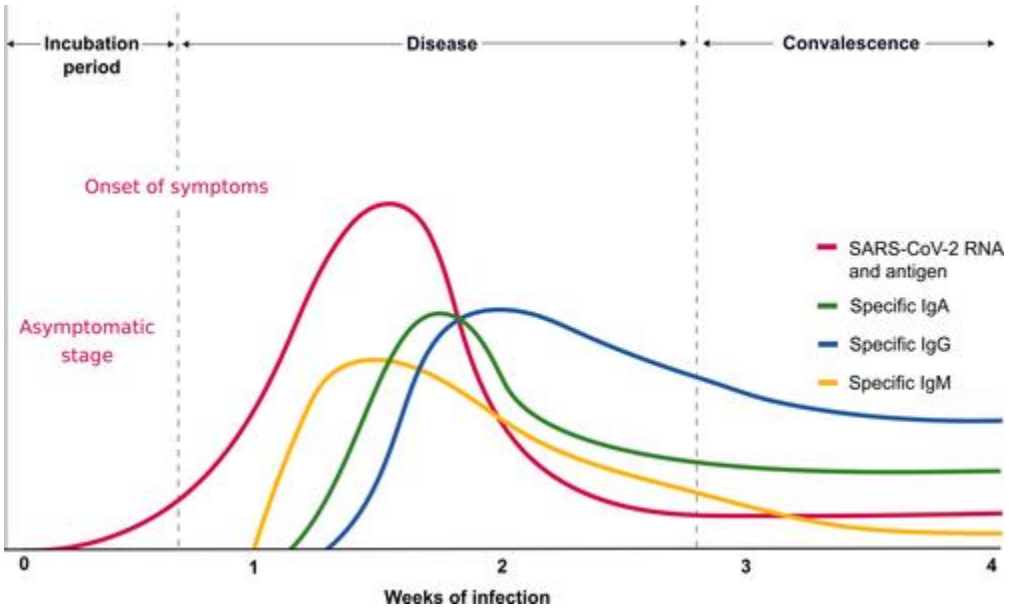


Figure 6: This figure describe of antibodies production and switching from the time of virus entering to the host cell until of recovery and long last immunity production against infection (Azkur *et al.* 2020).

1.6.1 Cytokine storm:

COVID-19 viruses attract the host cell through a process of clathrin mediated endocytosis(Umakanthan *et al.* 2020), during which the receptor binding protein

(RBD) of COVID-19 spike protein present on the surface of the viruses recognize the attachment of the angiotensin converting enzyme 2(ACE2) receptors which localized on the surface of target tissues mainly lung tissue and determined entry to the host cell (*Adil et al .2021)* ,which lead to intracellular viral replication until the cell bursts causing many virus particles to be released and then infect more and more cell that is leads to induction of immune system innate and adaptive one ,activation of immune system causing harmful damage due to cytokine storm which incorporated with the sever cases of COVID-19 patient that characteristic with many feature like dysregulated immune systemic response with sharply decrease count of CD4 T cell ,CD8 T cell ,b cell, natural killer cell ,monocyte ,eosinophil and basophil along with increase in neutrophils count , and also incorporated with elevated of pro-inflammatory cytokine including IL6,IL1Beta, IL2,IL8, IL17,G-CSF,GM-CSF, MCP1,TNF, all of these alteration are observed in the cytokine storm phenomena (*Adil et al.2021)* .

1.7 Diagnosis of COVID-19:

1.7.1 Diagnosis by Nucleic acid:

The speed and accuracy of diagnosis for COVID-19 patients is very important and helps control the pandemic and put in place emergency measures to prevent spreading and outbreak of the disease(Padoan *et al.* 2020).

The current method of Diagnosis of COVID-19 is based on nucleic acid RNA virological detection by real time polymerize chain reaction (RT-PCR) technique using swabs(naso and orapharengal) or deep sequencing , these method depends on the presente of the viral genome in sufficient quantities inside the sample that have been collected(Guo *et al.*2020), therefore , the improper way in sample collection can made usefulness of RT-PCR based assay and give as a false negative diagnosis which

can have a worsts consequences by allowing the infected patient who had diagnosed with negative to the presence of the virus to spread the infection (Guo *et al.*2020).

RT-PCR based on swabs to detect viral antigen is the current standard method of testing ,however 30% false –negative cases may be observed with this technique (*Adil et al .2021)* , it cannot be used to monitoring development of the disease at different stages and also cannot be applied to broad identification of previous infection and immunity (Carter *et al.*, 2020),in addition , real time polymerize chain reaction (RT-PCR) technique has some analytical and clinical limitations, RT-PCR exclude the ability to identifies patient who have been infected with minor or asymptomatic ,as a result they did not receive adequate treatment in adequate time (Padoan *et al.* 2020). That is what pulled us to search for another technique which can use it as a confirmatory one for the presence of COVID-19 and determined seroconversion of this virus with highest sensitivity and specificity at different stage of the disease and lowest consuming time and cost.

1.7.2 Diagnosis by Serological Testes :

In addition to RT-PCR technique, serological and immunoassay test are also recommended a supplement methods to RT-PCR with different pathogenic antigen target (Padoan *et al.* 2020). Additional serological diagnosis methods that can identified the presence of the virus regarding to the lower viral titer which made those method beneficial to ensure diagnosis of all infected patients and can be a technique that can be 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 identify 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).

Serological testing play an important role in monitoring and screening and also diagnosis for the presence of COVID-19 virus, in addition to assist in epidemiology and vaccine development by providing an verification whereas the infection is current or prior infection with COVID-12 virus , as well as antibodies diversity (Carter *et al.*, 2020). Most of the kits that use for antibodies and antigen screening test that depend on lateral flow immunoassays which is a diagnostic method used to confirm the presence or absence of a target antigen (Tsia et al .2021) .

Previously, immunoassay technique were used only for detecting presence of antibodies but in the recent years it has been also used to detecting of pathogen antigen by using primary monoclonal antibodies, the determination of COVID-19 is highly dependent on the detection of IgM or IgG antibodies specific to various viral antigen including, but not limited to, the spike glycoprotein (S1 and S2 subunits, binding receptors domain (RBD)) and a nucleocapsid protein, an example of these methods that are used to detect COVID-19 virus includes the traditional enzyme-linked immunosorbent assay (ELISA) which has high throughput capacity and less stringent specimen requirements than the RNA based assay (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),some of diagnostic test also may be contain flow-cytometer analysis for CD4+ and CD8+ T cell counts(Pal *et al.*2020), also Western Blot Assay can be used for detection of Antibodies against Coronavirus salian et al .2020).

1.7.3 Western Blot analysis:

Western blot analysis is a serological method uses to detected specifics protein and other translation modifications of that protein in the specimen and give us semi-quantitative or quantitative data about that specific target protein (Vallejo et al .2013).

Western blot contain a multiple steps, but not limited to sample preparation and protein extraction from the cell and separation of specific protein by SDS polyacrylamide page and transfer of target protein on nitro cellulose membrane , blocking of target protein on nitrocellulose membrane and loading of specimen with

specific antibodies and finally analysis of specific protein band by using a software (Mishra et al .2017) (figure Blow).

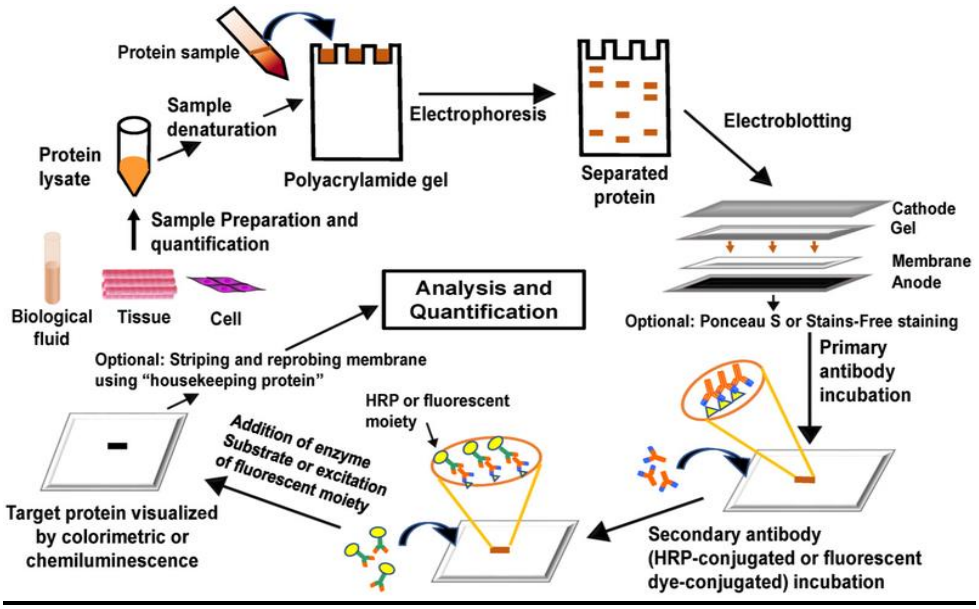


Figure 4: Schematic representation of the Western Blotting Procedure(Mishra et al .2017)

1.8 Treatment and vaccination:

The widespread of COVID-19 viruses all over the world has posed a wide challenge in the healthcare field (Sreepadmanabh et al. 2020) .There have been urgent calls for the development of rapid diagnostic tools and effective treatment protocols against the disease and, most importantly, vaccines against the causative agent of the disease(Sreepadmanabh et al. 2020).

1.8.1 Treatment strategies available for COVID-19 infections:

Conventional drugs: among the antiviral drugs based treatment chloroquine and its derivative hydroxychloroquine captured the most attention it is adenosine analog that inhibits the viral replication via works at the S protein of COVID-19 virus and that also used against Ebola, SARS-COV, and MERS-COV virus (Devaux et al .2020).

Plasma therapy: in addition to using drugs as a treatment of COVID-19 patients , plasma therapy is used also as effective practice treatment for COVID-19 patients, in

which it involves the use of plasma of the recovered patients as an adaptive immune therapy for critical cases (Sreepadmanabh et al 2020).

CP (covalence plasma) therapy is as an alternative therapy use to treat acute respiratory distress syndrome (ARDS) which are a complication of corona viruses disease in some critical case in which the principle of this method is depend on give the recipient patient blood plasma that is contain a high titer of antibodies against a pathogen from donor person who recovered from COVID-19 virus ,therefor provided an immunity boost to the recipient(Chen *et al.*2020).

There are an other emerging strategies instead the drugs that use as a treatment and limiting enter of the virus to the host cell, most of these strategies depend on designing a fusion inhibitor against COVID-19, spike protein an example of it HR2P fusion inhibitor against HR1 region of spike protein I addition to EK1 inhibitor (Xia *et al.* 2020).

Mesenchymal stem cell transplants for the COVID-19 patient also are clinically demonstrated, some of clinical study that clarified of transplant of stem cell to the COVID-19 patient that are lacked of ACE2 and TMPRSS2 gene expression made them have an effective immunity against SARS-COV-2 virus(Khoury *et al.*2020).

The development of many preventive factors against the COVID-19 virus is an important way in fighting the COVID-19 virus pandemic and its spread around the world(Sreepadmanabh et al 2020)

1.8.2 Vaccine of COVID-19 virus:

SARS-COV-2 virus is the emerged virus in this century which distribution all over the world and causes a pandemic disease called coronavirus disease 2019 (COVID-19) highly distribution rate of SARS-COV-2 virus and increase mortality rate of this viruses pulled health government and academic to urgently development an effective vaccine of that virus(Li *et al.*, 2020).

A group of vaccines targeting the COVID-19 virus has been developed and trial clinically(Li *et al.*, 2020). Some of the methods that have been proposed to vaccine development are:

Protein vaccine:

Protein fragment vaccine is depend on viral antigenic fragment produced by recombinant protein method , it is safely method to use and easily to production with a high tolerate rate in comparison to other viral vaccine in another way this type of vaccine it has a low immunogenicity(Li *et al.*, 2020), the development of protein subunit vaccine may be contain a full length of S protein based vaccine or s protein RBD vaccine this type of vaccine is characterized by immunogenicity and ability to developed highly protection against SARS-CoV-2 infection (Kam *et al.*, 2007; Li *et al.*, 2020), until now there is about 13 subunit of protein vaccines under the clinical trial for SARS-Cov-2 viruse among of them Novavax (NVX-CoV2373) vaccine enter phase III in trial(Li *et al.*2020).

Virus-like particle vaccine:

virus like particle vaccine is consist of whole structure that mimicry the real virus structure but lacked viral genome of the live virus which made them safe approaches of vaccine(Qian *et al.* 2020).

Viral vector vaccine:

it is a recombinant artificial virus that are holding the immunogenic antigen of the virus which is translate the virus antigen to the host cell like in a natural infection resulting in a robust immune response(Rauch *et al.*2018), Oxford–AstraZeneca COVID-19 vaccine developing by the oxford university is a viral vector vaccine (Rauch *et al.*2018).

DNA vaccine:

DNA vaccines consist of genes encoding viral immunogenic components that are expressed by plasmid vectors and transfer into cells through electroporation method(Rauch *et al.*2018).

RNA vaccine:

RNA virus vaccines consist of messenger RNA of the virus which translated to antigenic protein when entering the host cell that is lead to trigger the immune system response of the host , there are about 6 novel RNA vaccines Entered the clinical experience until the outbreak of COVID-19 virus such as Moderna and BioNTech/Pfizer both are a SARS-CoV-2 RNA vaccine(Kauffman et al. 2016).

Inactivated Whole virus Vaccine:

Whole cell inactivated vaccine are contain a particles of a bacteria or viruses that are killed chemically or radiologically to produce an inactivated virus with all immunogenic component of the original live virus(Li *et al.*, 2020), in convers to attenuated virus they did not become to reactivation when it enter to the host cell therefore they are safer than the live attenuated vaccine(Li *et al.*, 2020)

Live attenuated vaccine:

live attenuated vaccine consist of weekend or attenuated pathogen than causes the infection by muted the viral genome and it is similar to natural infection so it is provide a long term immune response furthermore live attenuated virus contain all virulence antigen of the nature virus so it is preserve the original conformation of the virus(Minor et al .2015).

1.9 Epitopes and Immune antigen response to COVID-19:

Once COVID-19 entr to the host cell attach with a specific cell that expressing a specific receptor in order to replication inside the infected host cell in case of COVID-19 virus mainly it located at respiratory epithelial cells(Azkur *et al.*, 2020). Until the COVID-19 enter and infected the host tissue viral peptides are presented by immune cell in specific MHC class I (major histocompatibility complex) of the CD8 cytotoxic T-cell leading CD8 cytotoxic cell to expansion in response to viral peptide antigen to produce memory cell and lysis tissue infected cell (Jansen *et al.* 2019).

In a few time of COVID-19 infection and after entering to the host tissue COVID-19 virus and all of it is particles are presented by MHC Class-II molecules that holed on

the immune cell mainly dendritic cell and macrophages to $CD4^+$ T cells which in turn to activation B cells to produce specific antibodies against the COVID-19 viral peptides(Jansen *et al.* 2019).

COVID-19 spike protein consist of S1 and S2 subunit which are cleaved at S2' cleavage sit and harbors a polybasic furin cleavage sequence (PRRARS) with an addition of 4 amino acid residue all of the memory T and B cell are production against COVID-19 structural protein S, N, and M proteins (Azkur *et al.* 2020). The observation of highly conserved epitopes sequence between COVID-19 and SARS-Cov-1 must be taken in considered(Grifoni *et al.*2020). Epitops is very short antigenic sequence determined a part of antigen to which the antibodies bind but generally is a piece of antigen that is recognized by immune system specially T and B cell(Azkur *et al.*2020).

Chapter two: Materials and Method

2.1. Serum collection:

A COVID-19 patient sera were collected from a confirmatory positive PCR patient, most of the serum samples were collected from Dura governmental hospital in Hebron city during December 2021, and this was under the supervision and ethical approval obtained from the Ministry of Health after formal cooperation has been established with hospital administrations that organized sample collection. Also 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.

The total number of collected samples were 60 samples, the collected serum samples were store on a refrigerator at -20 C^{o}. The collected sera samples serve for two main purposes: to have positive pooled serum for COVID-19 virus for initial phage and antigen selection to later individual sera samples to be used for antigen showing against profile of phage or recombinant antigen reactive against vaccinated or not vaccinated positive sera by western blot assay.

2.2. Antigen preparation (COVID-19 surface protein):

Based on previous work that was done in our laboratory it was possible to clone COVID-19 spike and membranes proteins in pET-28a expression plasmid and inserting this plasmid in Bcl2 cells by transformation. For this purpose c DNA was taken from a positive patient samples as a source of COVID-19 genetic material amplified by newly designed primers and cloned in suitable expression plasmids. These clones were designated as clone 105 (for COVID-19 spike protein and clone 8 for COVID-membrane protein). The exact methodology of cloning is not written in thesies as only these clones were used as source of antigen and the cloning was not done under this research.

2.3. Expression of COVID-19 recombinant surface protein:

For this purpose the following detailed procedure was used:

- A 500 ml autoclaved flasks was used for recombinant (Spike and membrane) protein expression, in each flask a 50ml LB media was added and then 0.5ml of Ampicillin and Kanamycin grown bacteria was added.
- Flasks were incubated for 2 hours at 37 C^o, with 200 rounds per minutes shaking in shaker water bath.
- After 2 hours, 50µl of Isopropyl-ß-D-thiogalactoside (IPTG) was added; IPTG is Allolactose-like chemical reagent that induces gene expression which it serves as an inducer, causing, and genes in the lac operon to start transcribed. Later, all flasks were kept at 37^{o}C for overnight incubation in the shaking water bath.
- In the next day, each flask was transferred to conical 50 ml tube and centrifuged at 5000 rpm for 10 minutes.
- The supernatant was decant and to the pellet 3 mL of phosphate buffer saline was added. This was the crude antigen preparation that was later more purified.

2.4. Purification of COVID-19 spike and membrane expressed proteins:

Purification of the His-tagged recombinant protein of COVID-19 procedure was done according to Novagen kit Nickle beads affinity chromatography protocol and as indicated by the manufacturer (Novogen, USA). The following is a summary of the used protocol:

- The precipitated bacterial cells in 3 ml phosphate buffer were lysed by sonication for about 10 minutes, then the lysed cells debris were separated from the extracted proteins by centrifugation at 5000 rpm for 10 min to separate the cell walls and other components, the supernatant which contain the desired proteins was collected in new tube.
- Nickle beads affinity chromatography matrix was used to purify His-tagged linked recombinant proteins as indicated in the manufacturer protocol, this is based on strong binding between the Histidine residues in the His tag attach with great specificity to the empty sites of the immobilized nickel ions on beads. Proteins were eluted by 0.1M concentration of phosphate buffer saline and kept at -20 C^o until further use.

The following is the list of the used buffers:

- 10ml 8X Binding Buffer:

(8ml 5M NaCl, 160ul 1M Tris, 40ul imidazole, 1.8 ml DDW)

2- 8ml 1X Binding buffer:

(Dilute the above buffer (7ml DDW and 1ml binding buffer)

3- 20ml 1X washing buffer:

(1ml 5 M NaCl, 40ul 1 M Tris, 40 ul imidazole, 8.9 ml DDW).

4- 10 ml 1X elution buffer

(1ml 5M Nacl, 20ul 1M Tris, 9ml 1M imidazole)

5- 10 ml 1X Charge buffer:

(50 mM NiSO4 in DDW) for activation beads.

2.5 M13 Phages display clones used in this study.

Identification and Purification of these clones was based the use of 12 amino acid Phage Display Library Kit # E8110S (New England Biolabs, Ipswich, MA, USA)**.** All the used protocols and reagents were as recommended by the manufacturer.

Based on previous work in our laboratory 10 different phage clones were picked, isolated and sequenced. The 12 identified amino acid in these clones showed (60-80%) similarity with COVID-19 epitopes, the identified epitopes were in a region of viral functional enzymes and structural, and based on NGS sequence analysis these clones were mixed: which means more than one M13 clones were picked, and some of these clones were not containing the 12 amino acid as part of the M13 epitope library. For this reason it was advised to further purify these clones against a new vaccinated positive COVID-19 in a step to have only the clones that may be related to COVID-19 spike protein and to have semi-pure phages that the selected epitopes will not be lost upon further culture and amplifications.

From the previous obtained M13 phage clones groups of clones were pooled, these clones showed the strongest reactivity with COVID-19 positive human sera. These clones were treated as the original M13 phage library (but these are enriched by COVID-19 epitopes). Individual specific reactive clones were re-picked from these pooled plaque clones and used in the current study. Based on this, two new groups were identified, they have the same 12 amino acid epitopes and they were mixed and

used as one clone. Namely these clones are: group A (plaques number 1 and 4), and group B (plaques numbers 2, 6, and 9).

Used reagents:

- Lauria broth (LB) preparation:

-(10g peptone, 5g yeast extract and 5g NaCl in 1L double distilled water), Autoclaved and stored at 4˚C until used.

- Top agar media **(**10g peptone, 5g yeast extract and 5g NaCl**),** dissolved in double distilled water then 7 gram of bacterial agar was added. Autoclaved and stored at 4˚C to be used

- Bacteria strains**:** E. coli XL1- blue bacteria

- IPTG\ X-gal preparation: 1M IPTG (Isopropyl-Beta-D-thiogalactoside) was prepared (2.3g IPTG was dissolved with 10ml double distilled water)

- TBS (Tris-bufferd saline) preparation: (50 mM Tris-HCl (pH 7.6), 150 mM NaCl). Autoclave, store at room temperature

- TBST (Tris-bufferd saline and Tween 20): 500ml TBST, (25ml Tris 7.5, 1.5ml 5M NaCl, 250 µl Tween-20 and 500ml double distilled water.

2.6. Picking of M13 reactive plaques with human Covid-19 positive sera:

Previously selected M13 plaques were re-amplified in XL1-Blue cells as indicated in the kit instructions. Simply, 50 µl of phage mixture from group A and group B were mixed with 200 µl of freshly grown XL1-Blue bacterial cells (exponential phase) and the mixture was mixed in 3 ml of pre-warmed top agar in 15 ml tubes, mixed and quickly dispensed on LB agar plates. Plates were incubated for overnight at 37^{o}C. In the next day the, plates that were containing plaques were transferred into a nitrocellulose membrane. Membranes were prick labeled for proper plate and membrane ordination, and to be used later for picking of reactive clones.

The transferred plaques on the dried nitrocellulose membrane were tested for their specific reactivity against COVID-19 vaccinated positive pooled sera. For this purpose the dried membranes were submerged in phosphate buffer saline for few minutes and then blocked with 5% FCS in PBS with Tween-20 for 30 minutes.

Finally, screened by COVID-19 vaccinated positive pooled sera as mentioned later in Western blotting.

2.7. Amplification and sequence analysis.

The DNA of the newly selected M13 plaques were amplified by polymerase chain reaction amplification and later sequenced by Sanger DNA analysis. The PCR reaction was carried out in 25 µl containing (2x ready mix Taq DNA polymerase mixture (Takara, Japan), 10 pmoles direct and reverse primers (Ph D1 (TTA TTC GCA ATT CCT TTA GTG) and Ph R2 (TCC CTC ATA GTT AGC GTA ACG), and 5µl DNA were added to each PCR reaction, double distilled water was used to fill the reaction volume up to 25µl. The used thermal profile in thermo-cycler involving 5 min at 95°C to initial denaturation, followed by 35 cycles each of has 30 second at 95°C to complete denaturation, 30 sec at 53°C Tm to perform annealing, followed by extension step (elongation) that involved 1 min at 72°C, and a final elongation step at 72°C for 10 min. The expected band size was estimated to be of 245 base pairs.

2.8. Agarose gel electrophoresis and DNA purification:

The amplified samples were run on agarose gel electrophoresis, for this purpose an agarose gel was prepared (1.7% agarose were dissolved in 100 ml 1x TAE buffer (50X TAE electrophoresis running buffer containing: 242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (pH 8.0)). Then 10µl of Ethidium Bromide was added for DNA staining. After gel solidifying, 10µl of each amplified clone was loaded to each well. An electric current was applied (120 voltage for 45 minutes). DNA size marker of 100bp DNA ladder (Thermos Scientific, USA) was used.

The amplified PCR products of the phage amplified clones were purified uisng Qaigene PCR purification kit (Qiagen, Germany) and as indicated by the manufacturers' instructions. Simply, DNA purification was done as the following: To 15µl PCR product from each sample 100µl of double distilled water was added, and to this 200µl of binding buffer was added (all mixtures were done iin 1.5ml eppendorf tube. The tubes were centrifuged for 60 second to discard flow- through. Then the bound DNA was washed twice with 700µl wash buffer (provided by the kit), in each

time columns were spined for about 30 second. This was followed by 1 minute continuous centrifugation to completely dry the membranes, directly the column was transferred into 1.5ml microcentrifuge tube followed by the addition of 50 µl double distilled water to elute the bound DNA. The purification column (elution tube) was discarded and purified DNA was stored at -20^{o} C to be used for DNA sequence analysis.

2.9. DNA sequencing:

Purified PCR products were sequenced according to dye terminator method, using Automated DNA Sequencer machine (AB477). The primers that used to DNA amplification were used there, for each PCR product direct primer was used in DNA sequences acquaint.

2.10. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE).

The buffer of the protein solution must be compatible with that of the gel separation method, for Tris/glysin SDS-polyacrylamide gel the PH of the protein solution must be approximately neutral and the salt concentration should be below 200 mM. COVID-19 antigen was prepared for SDS-PAGE gel electrophoresis and Western blot analysis according to the following procedure:

The prepared semi-purified protein samples (from clone 105, 8 and from phage clones) were diluted in sample buffer (loading buffer) for SDS-polyacrylamide gel electrophoresis. The loading buffer contains (2% SDS, 100 mM dithiothreitol,60mM Tris (PH 6.8) and 0.01% bromphenol blue) that is normally added to the sample from a 2x stock. The mixed sample and the loading buffers were boiled for 5 minutes before loading on acrylamid gel.

SDS-PAGE Gel Electrophoresis (SDS-PAGE): SDS-PAGE electrophoresis method was used for separating complex mixture of protein or polypeptide according to their molecular size.

Reagents and buffers were used for SDS-PAGE:

1-Running gel (lower gel):

9.6ml H2O, 5 ml 40% acrylamide mix, 5ml Tris PH(8.8) ,200 µl 10% SDS(sodium dodecyl sulfate) , 200 µl 10% APS(ammonium persulfate) ,10 µl Temed (N,N,N',N'-tetramethylethylethylenedaimine) .

2-Stacking gel (upper gel):

9.8ml H2O, 1.7 ml 40% acrylamide mix, 1.25ml Tris PH(8.8) ,100µl 10% SDS(sodium dodecyl sulfate) , 100 µl 10% APS(ammonium persulfate) ,10 µl Temed (N,N,N',N'-tetramethylethylethylenedaimine) .

3- 10% APS (ammonium persulfate) preparation:

1 gram of ammonium persulfate powder was dissolved in 10 mL distilled water

4-10% SDS (sodium dodecyl sulfate) preparation:

1 gram of SDS (sodium dodecyl sulfate) powder was dissolved in 10 mL distilled water

5-Runing buffer:

For 1 litter of distilled water (0.05 M Tris ,0.384 M glycine , 0.1% SDS)

Procedure for SDS-PAGE preparing: Samples to be tested on SDS-PAGE was diluted with buffer called loading buffer for Tris/glysin (2% SDS,100mM dithiothreitol,60mM Tris (PH 6.8) and 0.01% bromphenol blue),

- The running gel it was poured between the glass plates

-The lower gel was covered with a thin layer of (distilled water(D.W)) and then it was leaved to polymerize.

- The upper water was removed then the stacking gel was added and it was left to polymerize for about 20 minutes.

- The glass plates containing the gel was fixed on the electrophoresis set and filled with running buffer and connected to power supply.

- The sample protein with loading buffer was loaded in the wells; MW marker was added in each gel.

- Electrophoresis was carried out at 50 voltages until the bromophenol blue reached the running gel then at 120 voltages until the bromophenol blue reaches 1 cm before the end of the running gel.

2.11. Coomassie Brilliant Blue Staining:

Polyacrylamide gels were stained with coomassie brilliant blue stain (1g coomassie blue R250, 225ml methanol, 225ml acetic acid and 100ml distilled water) for 15 min and de-stained with de-staining solution (400 ml D.W, 50ml methanol and 50ml acetic acid). De-staining was done until obtaining clear bands on acrylamid gels.

2.12. Transfer of protein from gels to nitrocellulose membranes:

After protein mixture was resolved by SDS-PAGE analysis this was followed by the electro-transfer of protein from polyacrylamide gel onto nitrocellulose membrane (Whatman, USA), proteins were bonded strongly to nitrocellulose membrane which more accessible to antibodies than inside a polyacrylamide gel.

Buffers that were used for protein transfer:

-Transfer buffers: (0.025M TRIS-HCL ,0.192M Glycine, 20% methanol) This buffer was prepared by dissolved 10.5 gram Tris-Hcl and 50.4 gram Glycine in 2.8L of distilled water, and then the solution was completed to 3.5 L with absolute methanol solution.

- Ponceou-S Staining: (0.1% Ponceou-S, 50% glacial acetic acid in aqueous solution) which can be reused**.**

The separated proteins by SDS-PAGE gel were transferred into nitrocellulose filter membrane as follow: The polyacrylamide gel was soaked into 1X transfer buffer (0.025 M Tris-HCl, 0.192 M Glycine, and 20% methanol). Then, the gel and the nitrocellulose filter membrane were firmly gathered together inside the gel holder cassette. The gel holder cassette was inserted into Mini Trans-Blot tank filled with 1X transfer buffer, and then the transfer was carried out at 100 Volts for one hour or at 40 Volts for overnight. Then, the membrane was stained by Ponceau S (Sigma, Sant Louis, USA) (0.1% w/v in 1% v/v acetic acid) for 1 minute followed by de-staining in phosphate buffer solution.

2.13. Western Blot analysis:

After transfer, the membrane was cut into strips and blocked with blocking buffer (100ml phosphate buffer saline-Tween-20 (PBS-T) and 5ml Fetal calf serum (FCS)) for 30 min at room temperature (RT). Then, Strips were incubated for 2 hours

at RT with shaking with diluted sera samples (the choice of the samples is as indicated in each sol experiment, since sometimes positive vaccinated sera were used, or positive non-vaccinated serum or negative sera were used. Unbound antibodies were washed 3 times each for 5 min with 3ml PBS-T (washing buffer). After that, secondary antibodies (protein A–HRP) (Jakson antibodies) or anti-human IgM-HRP (Sigma Aldrich, USA) diluted in PBS-T (washing buffer) (1:6,000) were added for 1 hour with shaking at RT. Excess secondary antibodies were washed off 3 times with PBS-T (washing buffer). Bound antibodies were detected with DAB substrate (3, 3', 4, 4'-tetraaminobiphenyl), (10mg DAB, 15µl H_2O_2, 15ml 50Mm Tis-Hcl). Strips were incubated for 15-30 min at RT until band color was observed.

Reagent for immunoblotting:

- Phosphate buffer saline: (PBS 4 mM KH2Po4, 16 mM Na2 HPO4, 0.115M NaCl PH 7.3 (0.544 gram KH2PO4, 6.67 gram NaCl dissolved in one Liter of distilled water).
- Phosphate buffer saline with Tween-20 (TPBS) (Washing buffer): (1 Liter of (PBS) phosphate buffer saline with 500µl Tween-20.
- Blocking buffer or antibodies dilution buffer: (5% FCS (fetal calf serum) dissolved in (1 X PBS phosphate buffer + Tween)).
- Substrate solution: (6 mg of DAB (3,3',4,4'-tetraaminobiphenyl)(Sigma) were dissolved in 10 ml of 50 mM Tris with pH 7.5 and 100µl of H_2O_2)
- Protein A secondary antibodies : binding human IgG antibodies acting as anti-human conjugated to Horseradish Peroxidase (HPR), and it was diluted (1:6000) with antibodies dilution buffer (PBST + FCS).

Chapter two: Results

3.1. Purification of Spike and Membrane antigens.

As it was indicated in material and methods, a previously cloned spike and membrane COVID-19 proteins were expressed in Pet-28a expression plasmid vector and purified using nickel beads. From each clone, it was possible to prepare at least 5 ml of 1mg/ml of the indicated antigens dissolved in phosphate buffer saline, ready to be used in Western-blot analysis and indicated below.

3.2. Identification of new M13 phage display clones.

In this part a standard protocol was used to screen the already available mixed M13 phage clones with COVID-19 vaccinated positive sera. These clones were identified as group A and group B. Since this research is concentrating on evaluating the already produced recombinant clones and M13 selected phages reactive with COVID-19 positive sera, then no big emphasis was given on the tools and results that were used in selection of M13 phage clones. This type of work was already done in the lab and the current research is dealing with more fine screening of the mixed M13 phage clones. Several plaques were obtained in two successive screening and selection of the two M13 group phages against COVID-19 positive vaccinated sera.

In a summary, at the end of the screening 10 clones were selected, and they were subjected for DNA PCR amplification and sequence analysis. Figure 7, shows agarose gel electrophoresis of the PCR amplified individual plaques. For this, 10 individual plaques that are reactive with COVID-19 vaccinated positive pooled sera were picked and the internal 12 amino acids amplified with direct and reverse primers as indicated in materials and methods. The amplified bands were cleaned by PCR purification kit to remove all extra nucleotides and the reaction buffer, and then the 10 purified PCR bands were sequenced by Sanger method from these 10 different plaques we was selected 9 plaques .

DNA sequence analysis was successfully obtained for most of the analyzed plaques. The obtained sequences were aligned using Clustal Omega DNA alignment tools; this was done for 9 different plaques (Figure 8). As shown in Figure 8, clones 1, 3, and 4 have the same 12 amino acid sequence epitope and they were grouped together. in fact only plaques 1 and 4 were grouped and they were named group A. On the other hand, clones 2, 6, and 9 also showed the same 12 amino acid sequence and they were grouped together as well and they were named group B.

So, both group A (plaques 1 and 4) and B (plaques 2, 6, and 9 plaques) were evaluated for their reactivity against different types of collected sera (COVID-19 positive vaccinated, COVID-19 positive non-vaccinated and negative serum samples). Also recombinant clones 105 and 8 that represent recombinant spike and membrane proteins were evaluated for their immunoreactivity against the collected serum samples.

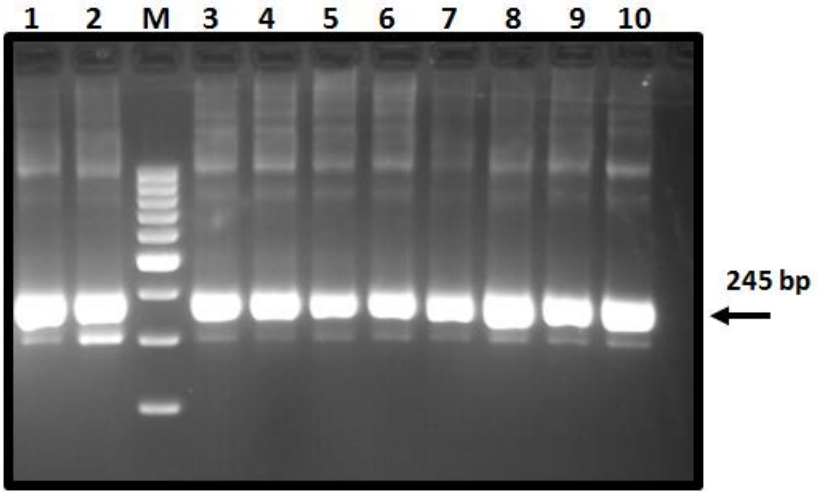


Figure 7: Agarose gel electrophoresis analysis of PCR products. The amplified band represents 36 nucleotides coding the 12 amino acids fused with the PIII M13 phage protein and together with the direct and reverse flanking region. 1-10: represents 10 selected and successfully amplified clones that were later sent for DNA sequence analysis. M: 100bp molecular weight marker.

```
Plaque8      TTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTGGTACCTTTCT      60
Plaque1      TTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTGGTACCTTTCT      60
Plaque3      TTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTGGTACCTTTCT      60
Plaque4      TTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTGGTACCTTTCT      60
Plaque5      TTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTGGTACCTTTCT      60
Plaque7      TTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTGGTACCTTTCT      60
Plaque6      TTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTGGTACCTTTCT      60
Plaque2      TTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTGGTACCTTTCT      60
Plaque9      TTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTGGTACCTTTCT      60
             ************************************************************

Plaque8      ATTCTCACTCTTATGATTCGTCGTTTACTGCTGAGGGGGATCCTCCGGGTGGAGGTTCGG      120
Plaque1      ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG      120
Plaque3      ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG      120
Plaque4      ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG      120
Plaque5      ATTCTCACTCTACTGCGAAGTATCTGCTTTGCGTCCTGGGCCGCTTTGGTGGAGGTTCGG      120
Plaque7      ATTCTCACTCTACTGCGAAGTATCTGCCTATGCGTCCTGGGCCGCTTGGTGGAGGTTCGG      120
Plaque6      ATTCTCACTCTACTGCTAGTATTGCTTCTGCTCATTTTGGTGGTTCTGGTGGAGGTTCGG      120
Plaque2      ATTCTCACTCTACTGCTAGTATTGCTTCTGCTCATTTTGGTGGTTCTGGTGGAGGTTCGG      120
Plaque9      ATTCTCACTCTACTGCTAGTATTGCTTCTGCTCATTTTGGTGGTTCTGGTGGAGGTTCGG      120
             **********  *              *                  ************

Plaque8      CCGAAACTGTTGAAAGTTGTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCT      180
Plaque1      CCGAAACTGTTGAAAGTTGTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCT      180
Plaque3      CCGAAACTGTTGAAAGTTGTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCT      180
Plaque4      CCGAAACTGTTGAAAGTTGTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCT      180
Plaque5      CCGAAACTGTTGAAAGTTGTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCT      180
Plaque7      CCGAAACTGTTGAAAGTTGTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCT      180
Plaque6      CCGAAACTGTTGAAAGTTGTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCT      180
Plaque2      CCGAAACTGTTGAAAGTTGTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCT      180
Plaque9      CCGAAACTGTTGAAAGTTGTTTAGCAAAATCCCATACAGAAAATTCATTTACTAACGTCT      180
             ************************************************************
```

Figure 8: DNA alignment of the PCR sequenced plaques. The sequences shows a typical 100% aligned DNA sequences from both direct and reverse ends and the coding region of the 12 amino acids in each specific plaque. Plaques were grouped as group A (1 and 4 plaques), and group B (plaques 2, 6, and 9).

3.3. Reactivity of recombinant clones and selected plaques with human sera.

In previous studies that were done in our laboratory both the recombinant spike and membranes proteins were evaluated for their reactivity with COVID-19 positive sera using ELISA test only. For better evaluation of these antigens it is recommended to test their reactivity in Western-blotting analysis. Figure 9, shows an initial trail for the reactivity of three used antigenic preparation of this study (recombinant spike protein, recombinant membrane protein, and a mixture of M13 plaques with COVID-19 reactive epitopes). These antigenic preparations were tested once as they extracted from the culture supernatant (named sup) and another as they were extracted from the precipitated bacterial culture (named ppt). The analysis was performed by Western-blot analysis using COVID-19 pooled positive sera as first antibody and protein-A/ HRP conjugated as a second antibody, (Figure 9).

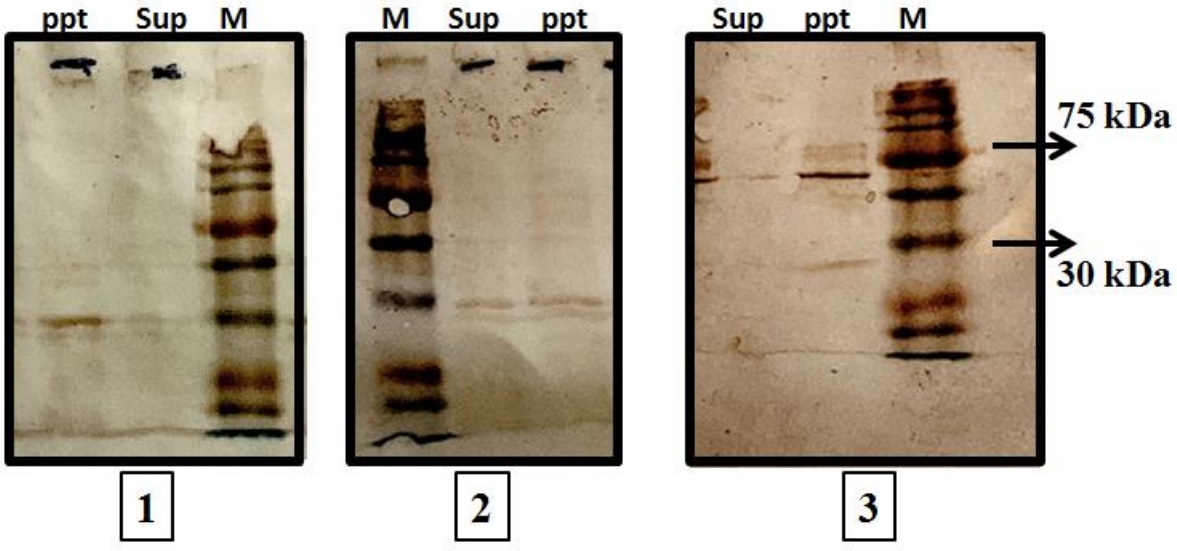


Figure 9: Western-blot analysis using three antigen preparations (both as sup and ppt antigenic extract) and Covid-19 positive human pooled sera as first antibody. (M: MW size marker) (ppt: precipetatnt) (sup: supernatent).

Based on the results of figure 9, it clearly shows that there is an interaction between the different antigenic preparations and the COVID-19 positive pool serum. The Western-blot shows a better reactivity with the 12 amino acids epitope bound M13 phage, in fact this preparation was a mixture of group A and group B plaques that were also reactive with COVID-19 pooled positive sera. The reactivity of the recombinant antigen showed a weaker interaction than the plaque antigenic mixtures. Also it was clearly seen that supernatant antigenic preparations contain less antigens compared to the antigen extracted from the precipitated bacterial cells, and this is true for all three antigenic preparations. So, based on this results it was decided only to use antigenic extract from the bacterial precipitated cells and not from the supernatant. The rational of these tests was to obtain more purified antigenic preparation specifically if we avoided starting the antigenic extract with the precipitated bacterial cells.

3.4. **Evaluating the reactivity of different antigenic extract against different status of COVID-19 collected sera.**

During serum sample collection it was possible to identify different serum samples that represents different clinical/vaccine status of patients that are infected by COVID-19. It was possible to have samples that were obtained from only vaccinated individual but never were infected by COVID-19 and other samples that were obtained from infected individuals without vaccination. The vaccinated serum samples also were possible to have them as those individuals whom received pFizer vaccine or other type of vaccine.

In the following part of this current research evaluation for the usefulness of the different antigenic preparations and the identified 12 amino acid epitopes was done. The first trail was done with 6 serum samples that were collected from individual with positive COVID-19 infection, some of them were vaccinated (there is no clear information on this for those individuals), one individual was with acute

infection of COVID-19 (not previously vaccinated). Figure 10, depicts the results of this pilot experiment in which western-blot analysis was done over the four different antigenic preparations (Group A 12 amino acids epitopes, Group B 12 amino acid epitopes, Spike recombinant protein, Membrane recombinant protein).

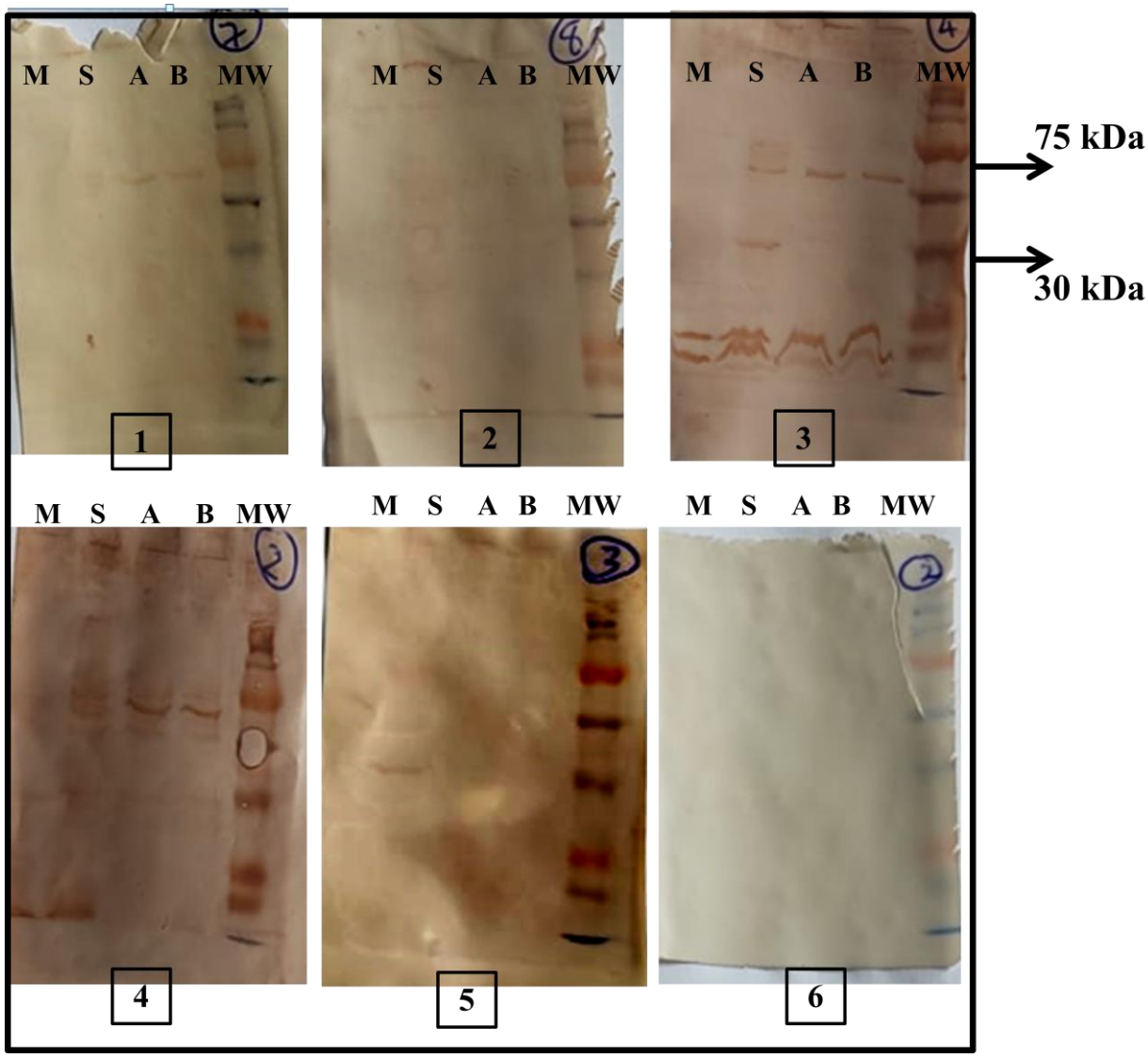


Figure 10: Western blot analysis using different antigenic preparations. (M: Membrane recombinant protein, S: Spike recombinant protein, A :group A 12 amino acid epitopes, B: group A 12 amino acid epitopes). 1-5: COVID-19 postive serum samples, 6: Acute COVID-19 infection.

The above results show a successful immunological reaction obtained by most of the used COVID-19 serum samples. These samples were not identified if those individuals were vaccinated or not .The shared criteria that they were COVID-19 antigen positive cases. Serum sample number 6, was known to be an acute case with a recent identified COVID-19 infection. The reactivity of the 12 amino acid epitopes with the different positive serum samples was more prominent compared to spike and membrane antigenic preparations (Figure 10).

In order to better understand the reactivity of the different antigenic preparation with different COVID-19 serum samples, we decided to group the serum samples into three main groups: 1- 10 samples that were characterized as positive and vaccinated with pFizer vaccine. 2- 10 samples that were characterized as positive and vaccinated with a vaccine other than pfizer. 3- 10 samples than were positive but not vaccinated. 4- Negative serum samples, that were obtained from old collected serum samples before year 2018 and they were proven to be stored at -20^{o}C since there collection. It is worth to mention that, in all the Western-blot analysis, protein A-HRP conjugated was used (as an indication for the presence of secondary human immune response represented by IgG antibodies). Only where it is indicated we used anti-humna IgM-HRP conjugated as secondary antibody in the Western-blot analysis.

This comparative evaluation study is depicted in figures 11-15. The figures are presented later as continuous pages in order to make easier to be followed and to be compared. In short we tested the immuno-reactivity of different COVID-19 serum samples with their different characteristics as indicated above (1-4), against the different antigen (Group A 12 amino acids epitopes, Group B 12 amino acid epitopes, Spike recombinant protein, Membrane recombinant protein). Figure 11 shows the results of western blot analysis using group A and group B 12 amino acids upon the use of vaccinated infected COVID-19 sera. Although there was differences inthe reactivity of different used serum samples to the 12 amino acid epitopes, but in general most of the serum showed a strong interaction with this type of antigen. These serum samples are vaccinated and infected, which reflects an immunological reaction that is equivalent to secondary immune responses with high IgG antibody titers. Also it was noted that group B 12 amino acid epitopes were more reactive than group A 12 amino acid epitope (Figure 11). Evaluating the usefulness of group A and B 12 amino

acids for their reactivity with infected but vaccinated, and this time other types of pFizer vaccine, it was seen that less reactivity of these epitopes to the tested individual sera was obtained (Figure 12). From 10 used serum samples (positive vaccinated with other than pFizer); only two samples gave a positive results in Wester-blot analysis. It is difficult to conclude if these vaccines were not effective enough to produce a type of a secondary immune response, or the used antigens are not cross-reactive with the used 12 amino acid epitope.

Surprisingly, the reactivity of spike and membrane recombinant antigen proteins with vaccinated infected samples was seen to be less than the reactivity of group A and B 12 amino acid epitopes with the same vaccinated infected samples (Figure 13). From the 10 used serum samples only 3 samples (sample: 3, 6, and 7) showed faint reaction in Western-blot analysis and only with the spike recombinant proteins. Based on previous studies in our laboratory we also found that these recombinant proteins are varied in their reactivity to different positive COVID-19 serum samples (This was done by ELISA assay).

In this study it also was possible to study the reactivity of 10 infected but not vaccinated serum samples against group A and B 12 amino acid epitopes (Figure 14). The obtained results in this aspect showed, revealed the reactivity of only 4 samples (namely: sample 2, 4, 6, and 7) with the 12 amino acid epitopes, mainly the B group (Figure 14). These samples could be of different timing from acute infection, and the severity of the current infection is not known, so it is difficult to judge about non-reactive samples with the 12 amino acid epitope, and whether if there is weak antibody titers or simply no antibodies are produced against these epitope parts.

Figure 15, shows the reactivity of the different antigenic preparations with previously collected negative COVID-19 samples, this is to show that there is no non-specific immunological interactions with these antigenic preparation.

The identification if these antigenic preparations could interact with primary produced antibodies (IgM) also was evaluated. Figures 16 and 17 shows the reactivity of group A and B 12 amino acids epitope regions with individual collected infected serum samples as well as their reactivity with serum samples that were collected from vaccinated individuals. In this Western-blot the used secondary antibody was anti-human IgM conjugated to HRP.

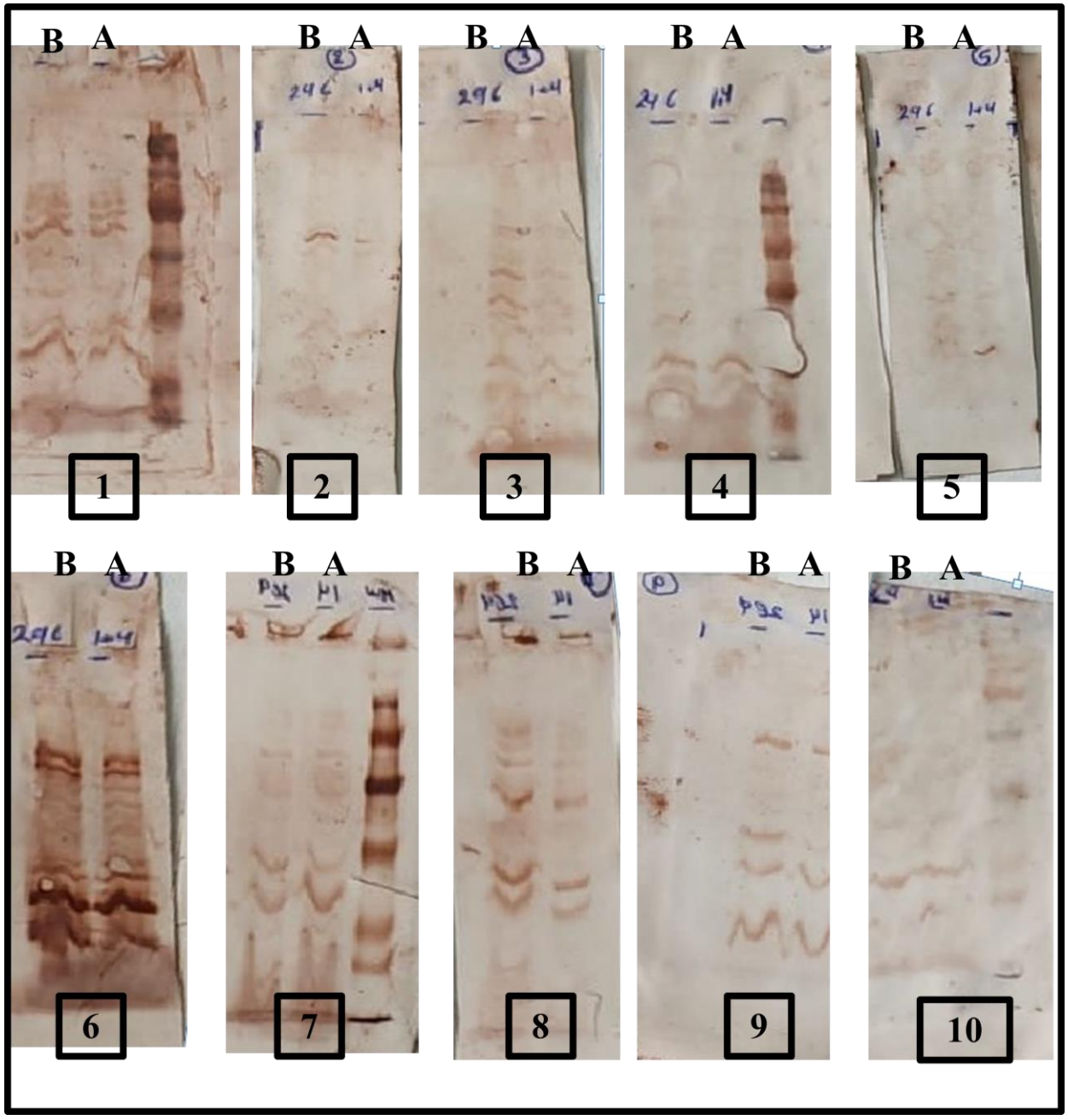


Figure 11: Western-blot analysis using group A and B 12 amino acids epitomic region against vaccinated (pFizer) infected serum samples.

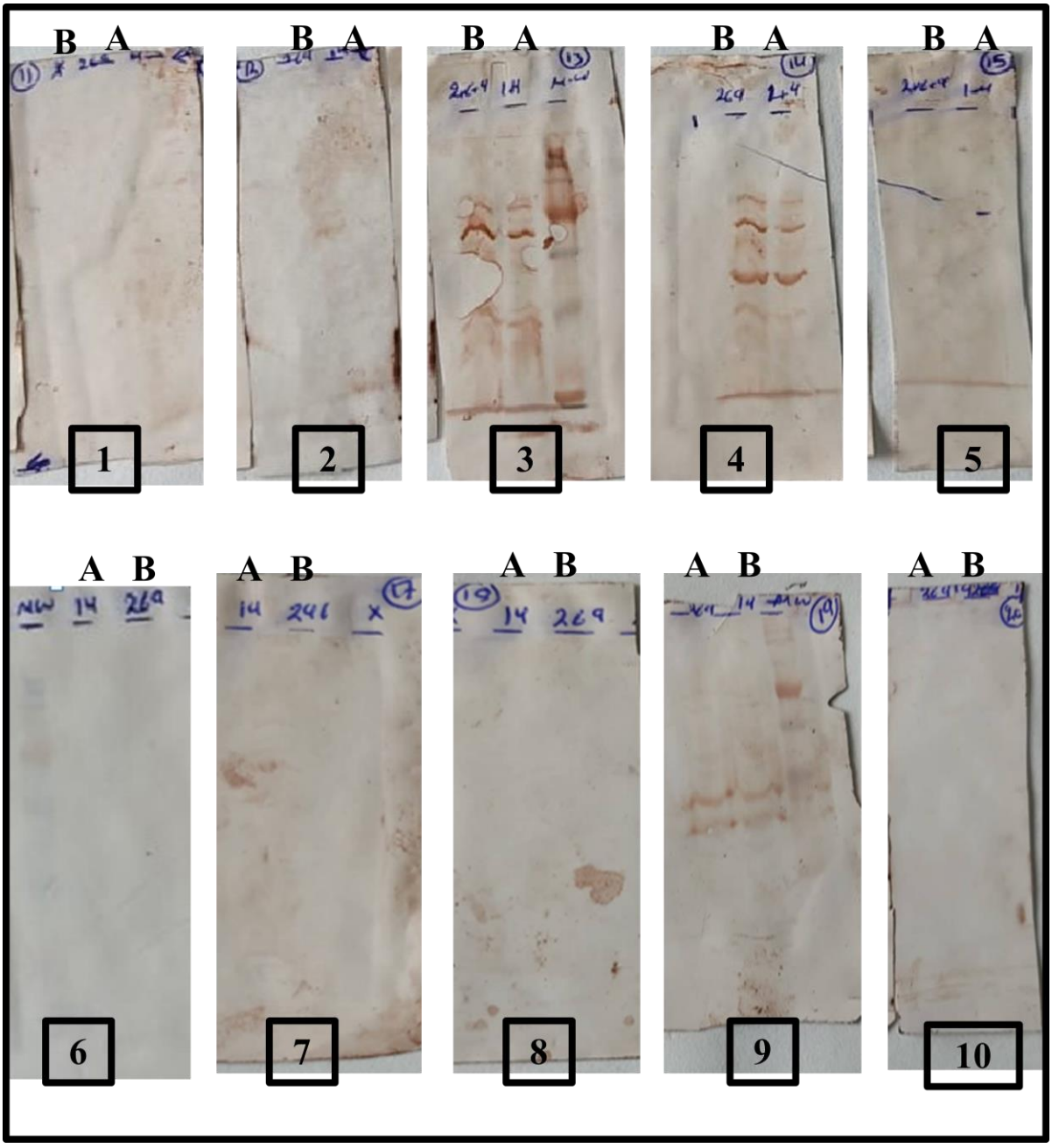


Figure 12: Western-blot analysis using group A and B 12 amino acids epitomic region against vaccinated (other than pFizer) infected serum samples.

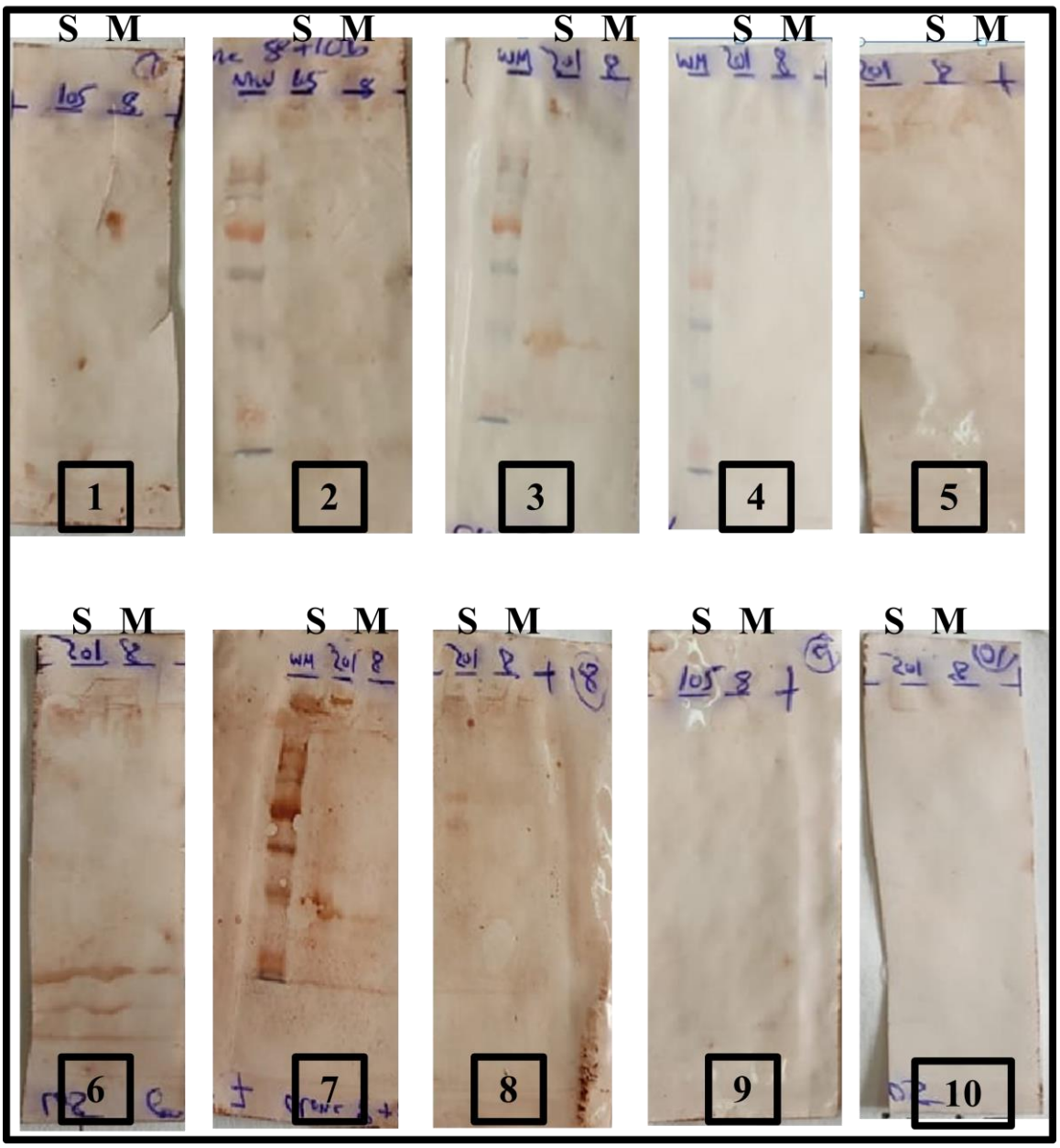


Figure 13: Western-blot analysis using spike and membrane recombinant proteins against vaccinated (pfizer) infected serum samples.

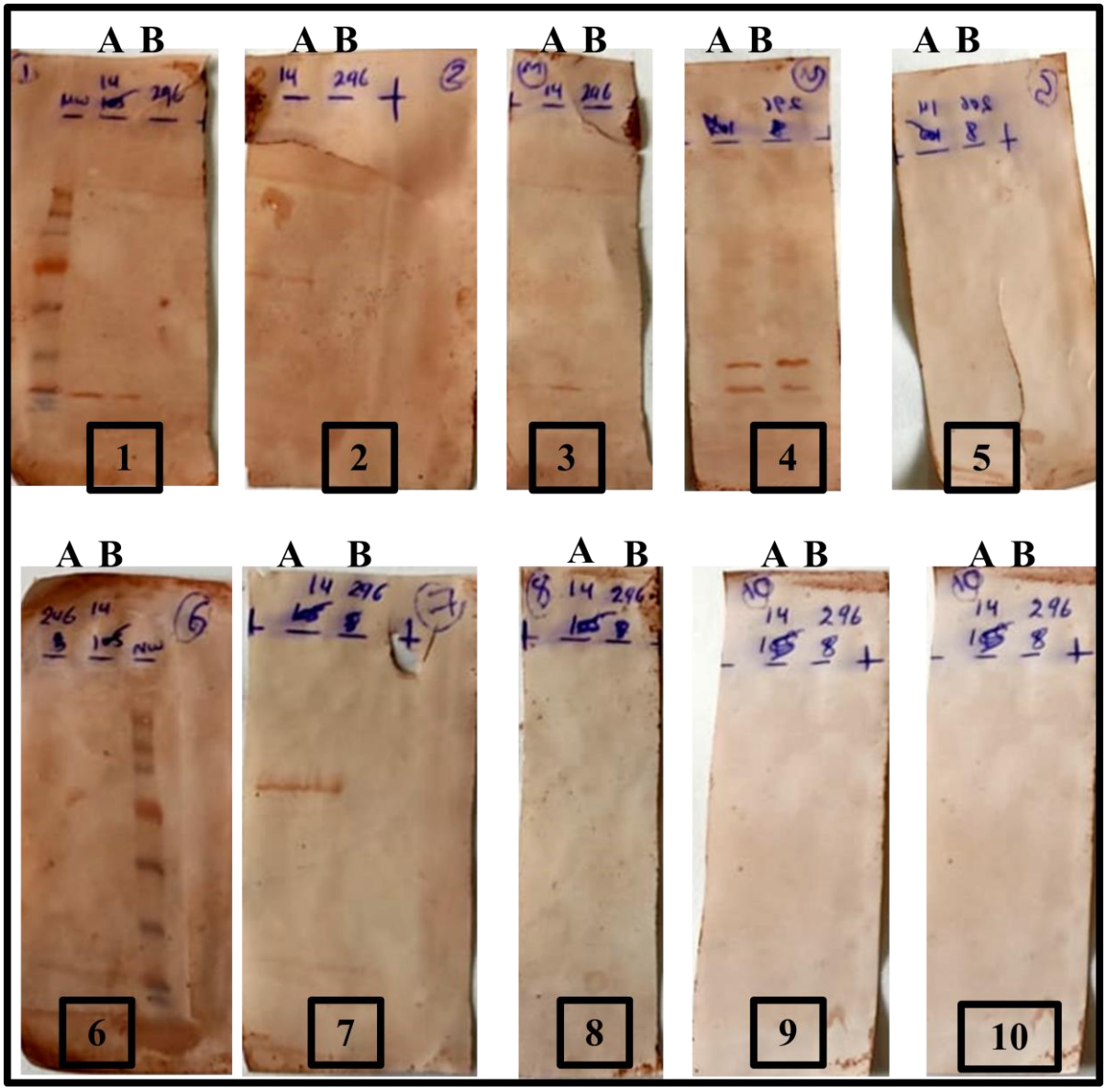


Figure 14: Western-blot analysis using group A and B 12 amino acids epitopic region against non-vaccinated infected serum samples.

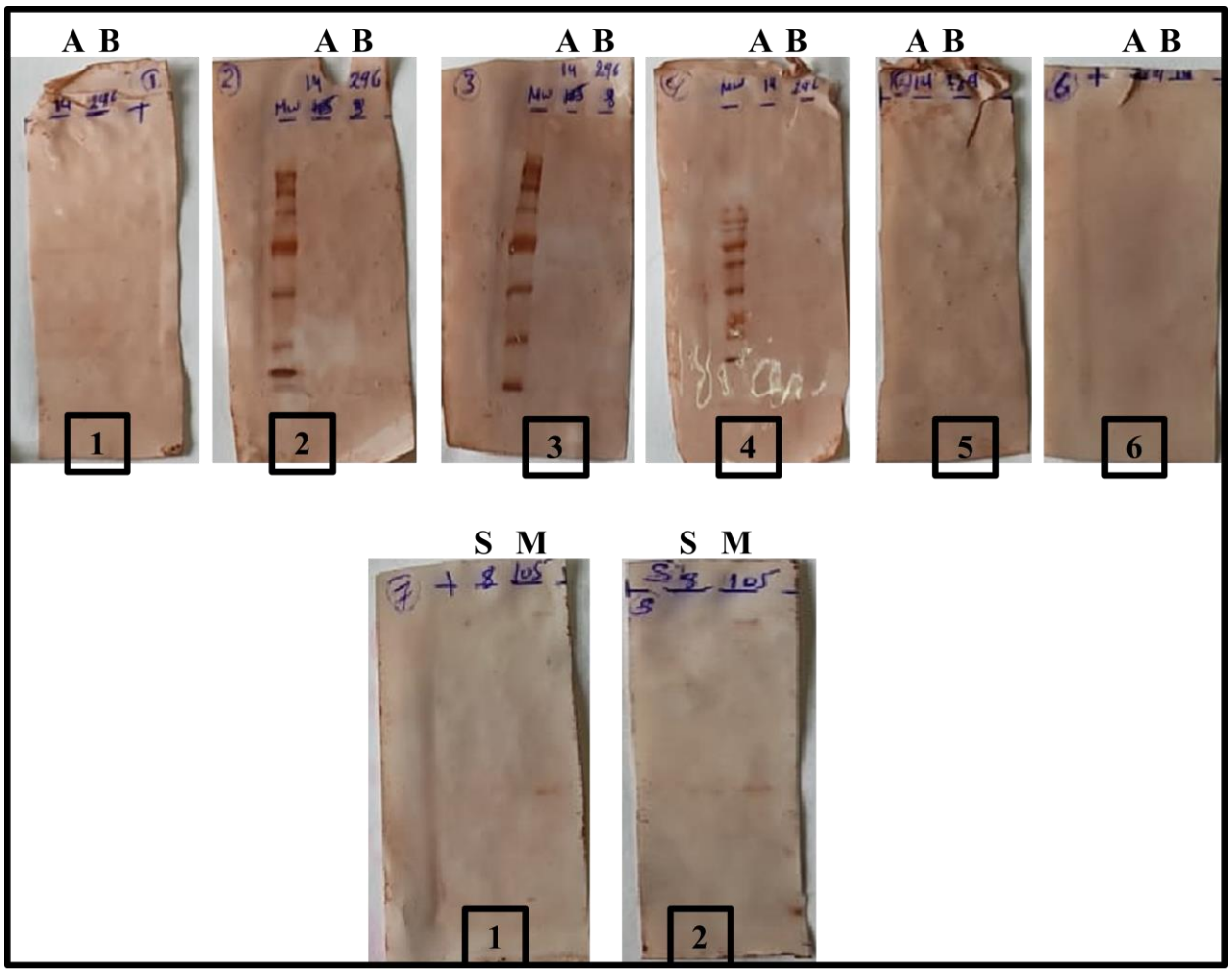


Figure 15: Western-blot analysis using group A and B 12 amino acids epitomic region and (spike and recombinant proteins) against COVID-19 negative serum samples.

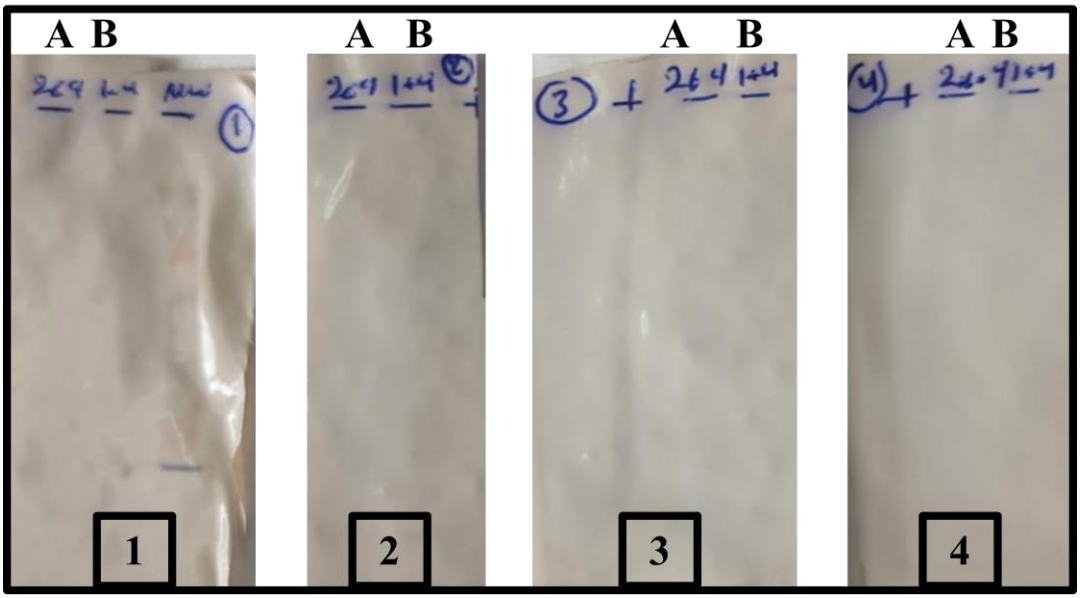


Figure 16: Western-blot analysis using group A and B 12 amino acids epitomic region against COVID-19 vaccinated serum samples. Anti-human IgM-HRP was used as a second antibody.

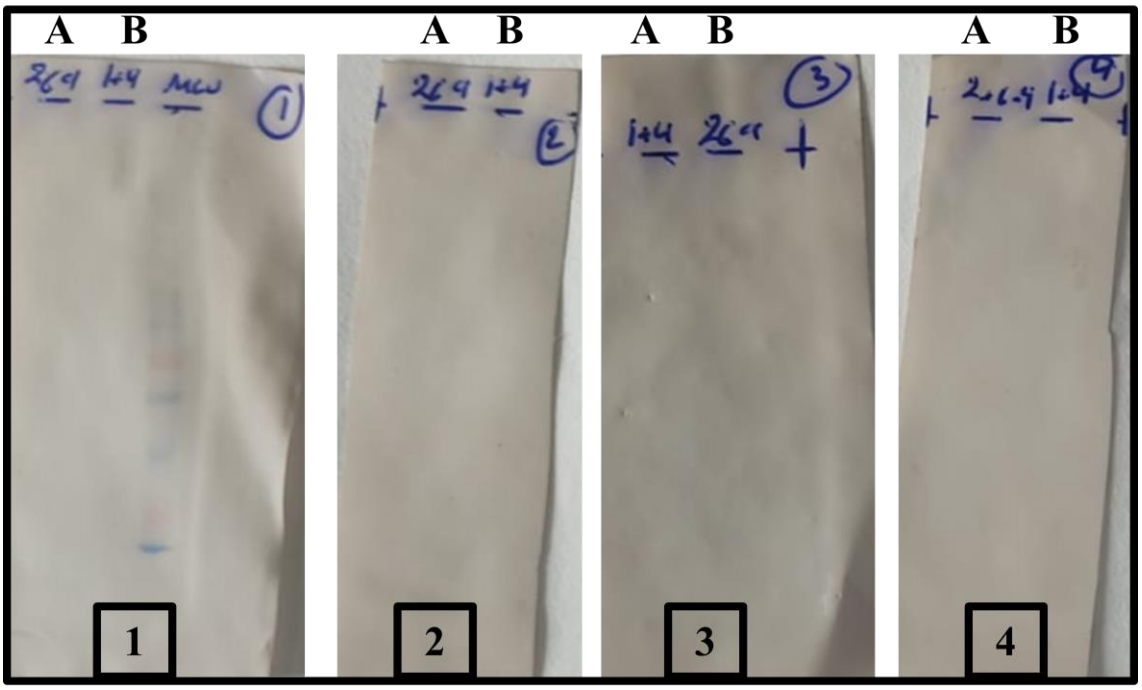


Figure 17: Western-blot analysis using group A and B 12 amino acids epitomic region against COVID-19 non-vaccinated serum samples. Anti-human IgM-HRP was used as a second antibody.

Chapter four: Discussion:

COVID-19 is a respiratory illness caused by the SARS-CoV-2 virus. It was first identified in Wuhan, China in December 2019 and has since become a pandemic, spreading to affect people in many countries around the world. Symptoms of COVID-19 can range from mild to severe and include fever, cough, difficulty breathing, and body aches. Some people may be asymptomatic or have very mild symptoms, but can still spread the virus to others. The virus is primarily spread through respiratory droplets produced when an infected person talks, coughs, or sneezes. It can also be transmitted by touching a surface or object contaminated with the virus and then touching one's face. There is currently no specific treatment for COVID-19, but research is ongoing to develop vaccines and treatments. In the meantime, supportive care can be provided to help manage symptoms and prevent complications.

The immune response to SARS-CoV-2, the virus that causes COVID-19, can vary among individuals. Some people may develop a strong immune response after being infected with the virus, while others may have a weaker response. In general, vaccines work by stimulating the immune system to produce antibodies and other immune cells that can recognize and fight the virus. This can provide immunity to the virus, either through direct protection or by boosting the immune system's ability to recognize and fight the virus. It is not yet known how long immunity from COVID-19 vaccines will last, but it is expected to be at least several months to a year. Some booster doses may be needed to maintain immunity over the long term. It is also not yet known how the immune response to COVID-19 vaccines compares to the immune response in people who have been infected with the virus. Some studies have suggested that the immune response to the virus may wane over time, while others have suggested that it may be more durable. More research is needed to fully understand the immune response to COVID-19 in vaccinated and non-vaccinated individuals.

The present study was performed to developed a suitable COVID-19 immunological test that can detect SARS-CoV-2 IgG and IgM antibodies in a qualitative and quantitative manner and to provide a continuous source of SARS-

CoV-2 surface protein antigenic epitopes in sufficient quantities using cloning technique and phages display method.

COVID-19 infection can be detected, characterized by the monitoring the evaluation of Secretory antibodies produced against virus antigens, When the COVID-19 virus binding with it is receptor ACE2(angiotensin converting enzyme 2) that was located inside the host cell, IgG and IgM neutralizing antibodies that was produced to prevent the infection to spread to non–infected cell. In the earlier day about the fifth day of infection a strong peak of IgM antibodies followed by IgG antibodies mostly on the 10 days after the onset of the disease(Fergie and Srivast 2021).

IgM and IgG are types of antibodies produced by the immune system in response to an infection or vaccination. They are proteins that are produced by B cells, a type of white blood cell, and are specific to a particular virus or other pathogen. IgM antibodies are typically the first to be produced in response to an infection. They are generally produced within a few days to a week after the start of an infection, and can be detected in the blood relatively soon after the onset of symptoms. IgM antibodies are usually present in higher levels at the beginning of an infection and then decline over time. IgG antibodies, on the other hand, are generally produced later in the course of an infection and can remain in the body for a longer period of time. They are often used as a marker of previous infection, as they can be detected in the blood long after the initial infection has resolved. After serological testing began to appear on a large scale many of these serological tests were not as accurate as the manufacturers claimed leading to an unacceptable number of false positive and false negative tests so consideration should be given to follow an initial positive serologic test result with a supplemental or confirmatory test having optimal specificity such as a Western blot(Pavia and Wormser 2020)

In the case of COVID-19, both IgM and IgG antibodies can be produced in response to infection with the SARS-CoV-2 virus. IgM antibodies may be detected in the blood within a few days to a week after the onset of symptoms, while IgG antibodies may take longer to appear and can remain in the blood for a longer period of time. The presence of IgM and IgG antibodies can be detected through a blood test called a serological test. These tests can be used to diagnose an active infection or to

determine whether someone has been infected with the virus in the past. They can also be used to monitor the immune response to the virus over time

For more than 25 years ago western blot was taken as a confirmatory test for HIV infection, so the proposed algorithm for confirmatory testing for measuring serologic reactivity due to COVID-19 would rely on using the Western blot technique (Pavia and Wormser 2020). A Reactive ELISA, or antibody immunoassay, will be followed by Western blot, which detects specific antibodies directed against various COVID-19 proteins(Pavia and Wormser 2020). Western blot is a laboratory technique that is used to detect and identify specific proteins in a sample. It is commonly used in the field of molecular biology and is often used to confirm the presence of a particular protein or to identify the specific proteins present in a sample. In the context of COVID-19, Western blot analysis can be used to detect and identify the presence of specific proteins in a patient's blood sample that are produced by the SARS-CoV-2 virus. These proteins, called antigens, can be used as markers to diagnose an active infection or to determine whether someone has been infected with the virus in the past. Western blot analysis can be used in conjunction with other diagnostic tests, such as PCR tests or serological tests, to help confirm the presence of COVID-19 in a patient. It can also be used to monitor the immune response to the virus over time and to track the progression of the infection.

In addition to shedding light on the development of preventive immunity and design of vaccines against the COVID-19 virus it is being proposed that the Western blotting technique be used to determine which antigen protein are determined in the serum from patients recovering from COVID-19 in order to identify components of the virus that should be part of a vaccine development (Pavia and Wormser 2020). For Western blot testing, standardize techniques are used in which purified viral proteins are separated by electrophoresis on gels then it is transferred onto nitrocellulose membranes and then reacted with the patient's serum that is diluted to the required concentration. The reaction is completed by treating the sample with IgM or IgG, (in some infection also IgA immunoglobin can be detected). therefore, Antibodies from the patient serum sample will bind to specific viral proteins which resulting in the visualization of bands based on their molecular weight and migration patterns along the gels (Pavia and Wormser 2020).

In this study, and after a purification of spike and membrane protein of COVID-19 and identification of M13 phages display clone that have been used as a COVID-19 antigen ,since this researches is concentration on evaluating the already produced recombinant clone and M13 selected phages that was reacted with a COVID-19 positive sera ,so this researches in general is more dealing with more fine screening of mixed M13 phages clone. After a DNA sequence analysis and alignment of the resulted plaques ,we was obtained of two different grouped of phages plaques each of them have the same 12 amino acid and called grouped A which contain plaques 1 and 4 and a grouped B of plaques that contain clone 2,6,9 of plaques both of them grouped (a and b) that were evaluated for their reactivity against a different type of COVID-19 collected sera (positive vaccinated ,positive non-vaccinated and negative serum samples). Also recombinant clones that represent recombinant spike and membrane protein were evaluated for their immunoreactivity a giant collected serum sample.

The western blot analysis of an initial trial for the reactivity of the three used antigenic preparation (recombinant spike and membrane protein and a mixture of M13 plaques) with COVID-19 reactive epitopes showed that there is an interaction between the different antigen preparation and COVID-19 positive pooled serum , and the best that for a mixture for a grouped A and grouped B of M13 phage plaques in contrast of the reactivity of the recombinant (spike and membrane) antigen which showed a weaker interaction with the COVID-19 positive pooled serum than the mixture of plaque. After that we were evaluating the immunoreactivity of different antigenic preparation against a different COVID-19 collected serum samples (infected not vaccinated with COVID-19 ,vaccinated against COVID-19 and a grouped of negative sample) it is should to be noted that the vaccinated COVID-19 grouped was divided into p-fizer vaccinated grouped and another grouped contain a patient serum sample that are vaccinated with other than p-fizer vaccine ,after a western blot analysis of this patient serum sample grouped with a different viral antigen we were prepared (spike and membrane recombinant protein and M13 phage plaque grouped A and B), the result was showed an immunological reaction that equivalent to a secondary immune response with a high IgG titers and also to be noted that the

grouped B 12 amino acid epitopes was more reactive than the grouped A 12 amino acid epitopes.

In this study it also was possible to study the reactivity of a grouped of infected but not vaccinated patient serum sample against a grouped A and B 12 amino acid epitopes these sample obtained from a different timing from acute infection and the severity of infection it is not known so it difficult to determine if there a weak antibodies titer or no antibodies are produced against these antigen epitopes part .

The SARS-CoV-2 virus, which causes COVID-19, has a surface protein called the spike (S) protein that is important for its ability to infect cells. The spike protein is responsible for binding the virus to host cells and allowing the virus to enter and infect the cells. The spike protein can be detected in the blood of individuals who have been infected with SARS-CoV-2, as well as in individuals who have been vaccinated against COVID-19. The presence of the spike protein in the blood can be detected through a laboratory test called a serological test, which is used to detect antibodies produced in response to the virus. In individuals who have been infected with SARS-CoV-2, the spike protein can be detected in the blood soon after the onset of symptoms. The levels of the spike protein in the blood may increase as the infection progresses and then decline as the immune system works to clear the virus from the body.

In individuals who have been vaccinated against COVID-19, the spike protein can also be detected in the blood. The presence of the spike protein may indicate that the individual has mounted an immune response to the vaccine and that the body is producing antibodies that are specific to the spike protein. The detection of the spike protein in the blood can be used to confirm the presence of COVID-19 infection or to determine whether someone has been vaccinated against the virus. It can also be used to monitor the immune response to the virus over time and to track the progression of the infection or the effectiveness of the vaccine.

The spike (S) protein is a surface protein found on the SARS-CoV-2 virus, which causes COVID-19. It is important for the virus's ability to infect host cells, as it allows the virus to bind to and enter host cells. Western blot is a laboratory technique that is used to detect and identify specific proteins in a sample. It can be used to detect the presence of the spike protein in the blood of individuals who have been infected with SARS-CoV-2 or who have been vaccinated against COVID-19.

In the case of COVID-19, Western blot analysis can be used to detect the presence of the spike protein in the blood of infected individuals. It can also be used to detect the presence of the spike protein in the blood of individuals who have been vaccinated against COVID-19, which may indicate that the individual has mounted an immune response to the vaccine and is producing antibodies specific to the spike protein. The detection of the spike protein using Western blot analysis can be used to confirm the presence of COVID-19 infection or to determine whether someone has been vaccinated against the virus. It can also be used to monitor the immune response to the virus over time and to track the progression of the infection or the effectiveness of the vaccine.

Conclusions and Recommendations:

In general the use of M13 phage display plaques containing 12 amino acids that were selected using COVID-19 pooled infected sera; were proved to be more reactive with vaccinated and even infected and non-vaccinated COVID-19 serum samples, this compared to the use of spike or membrane recombinant protein antigenic preparations. It was clearly seen that these antigenic preparations were only immunoreactivity with IgG and not IgM, which suggests their usefulness in detecting cases with high IgG antibody titers and never in acute COVID-19 infections. This finding could emphasis the potential of using the M13 phage display plaques containing 12 amino acids in evaluating vaccinations procedures against COVID-19.

ملخص باللغة العربية:

العنوان: استخدام تقنية ال western blot في الكشف عن وجود اجسام مضادة ل فيروس COVID-19

اعداد: أسيل باسم سامي اقنيبي

اشراف: د. رسمي ابو حلو، د. ابراهيم عباسي

مقدمة:فيروس كورونا هوعبارة عن فيروس يصيب الجهاز التنفسي ويطلق عليه اسم المتلازمة التنفسية الحادة (SARS-Cov-2) ، ينكون هذا الفايروس من جينوم RNA أحادي موجب الاتجاه (+ ssRNA) حيث يترواح حجمه بين ال ٣٠ و ال ٣٢ كيلو بايت تتضمن مجموعة من البروتينات السطحية والوظيفية ، يقوم فيروس كورونا ب احداث المرض والوصول الى خلايا الجسم من خلال ارتباطه بالخلايا السطحية المعروفة ب اسم ACE2 والموجودة في خلايا الرئة ، الهدف الرئيسي من هذه الدراسة هو استخدام الانتيجينات المختلفة لفيروس كورونا و التي تم الحصول عليها مخبريا لفحص وجود لاجسام المضادة داخل مجموعة من العينات الماخوذة من مجموعات مختلفة من المرضى ب استخدام تقنية الWestern-blot

الطريقة: تم استخدام مجموعة من ال plaques تسمى ال M13 phase plaques والتي تحتوي على ١٢ منطقة من الاحماض الامينية ضد مجموعة من الامصال الإيجابية المجمعة ل مرضى COVID-19، تم استخدام مجموعتان من ال ١٢ amino acids والبروتينات المصنعة في تحليل ال Western-blot، كانت الاصال المستخدمة في هذه الدراسة ثلاثة أنواع : (٢٠ عينة مصل COVID-19 موجبة ومطعمة ، ١٠ عينات مصل إيجابية غير ملقحة ، وبعض عينات المصل السلبية).

استنتاج النتائج: بشكل عام استخدام phages M13 التي تحتوي على ١٢ حمضًا أمينيًا تم اختيارها باستخدام الأمصال المصابة بفيروس COVID-19 ؛ ثبت أنه أكثر تفاعلًا مع عينات مرضى ال COVID-19 المحصنة وحتى المصابة وغير الملقحة ، وهذا مقارنة باستخدام مستضدات البروتينات المؤتلف السنبلة ال sprotein أو الغشائية ال m protein. وقد لوحظ بوضوح أن هذه الانتيجينات المختلفة لم تتفاعل سوى مع IgG وليس IgM ، مما يشير إلى فائدتها في الكشف عن الحالات التي تحتوي على عيارات عالية من الأجسام المضادة IgG وليس في حالات عدوى COVID-19 الحادة. يمكن أن تؤكد هذه النتيجة على إمكانية استخدام phages M13 التي تحتوي على ١٢ من الأحماض الأمينية في تقييم إجراءات التطعيم ضد COVID-19.

Reference:

Alanagreh, L., Alzoughool, F. and Atoum, M. (2020) 'The Human Coronavirus Disease COVID-19: Its Origin, Characteristics, and Insights into Potential Drugs and Its Mechanisms', *Pathogens*, 9(5), p. 331. Available at: https://doi.org/10.3390/pathogens9050331.

Amanat, F. *et al.* (2020) 'A serological assay to detect SARS-CoV-2 seroconversion in humans', *Nature Medicine*, 26(7), pp. 1033–1036. Available at: https://doi.org/10.1038/s41591-020-0913-5.

Approaches towards fighting the COVID-19 pandemic (Review) - PubMed (no date). Available at: https://pubmed.ncbi.nlm.nih.gov/33236131/ (Accessed: 18 October 2021).

Azkur, A.K. *et al.* (2020) 'Immune response to SARS-CoV-2 and mechanisms of immunopathological changes in COVID-19', *Allergy*, 75(7), pp. 1564–1581. Available at: https://doi.org/10.1111/all.14364.

Carter, L.J. *et al.* (2020) 'Assay Techniques and Test Development for COVID-19 Diagnosis', *ACS Central Science*, 6(5), pp. 591–605. Available at: https://doi.org/10.1021/acscentsci.0c00501.

Castro Dopico, X. *et al.* (2022) 'Immunity to SARS-CoV-2 induced by infection or vaccination', *Journal of Internal Medicine*, 291(1), pp. 32–50. Available at: https://doi.org/10.1111/joim.13372.

Chen, L. *et al.* (2020) 'Convalescent plasma as a potential therapy for COVID-19', *The Lancet. Infectious Diseases*, 20(4), pp. 398–400. Available at: https://doi.org/10.1016/S1473-3099(20)30141-9.

Chowdhury, M.A. *et al.* (2020) 'Immune response in COVID-19: A review', *Journal of Infection and Public Health*, 13(11), pp. 1619–1629. Available at: https://doi.org/10.1016/j.jiph.2020.07.001.

COVID-19: A Global Challenge with Old History, Epidemiology and Progress So Far - PubMed (no date). Available at: https://pubmed.ncbi.nlm.nih.gov/33374759/ (Accessed: 18 October 2021).

COVID-19 Vaccines May Not Prevent Nasal SARS-CoV-2 Infection and Asymptomatic Transmission - Benjamin S. Bleier, Murugappan Ramanathan, Andrew P. Lane, 2021 (no date). Available at: https://journals.sagepub.com/doi/full/10.1177/0194599820982633 (Accessed: 18 October 2021).

Cui, J., Li, F. and Shi, Z.-L. (2019) 'Origin and evolution of pathogenic coronaviruses', *Nature Reviews. Microbiology*, 17(3), pp. 181–192. Available at: https://doi.org/10.1038/s41579-018-0118-9.

Development of a Western blot assay for detection of antibodies against coronavirus causing severe acute respiratory syndrome - PubMed (no date). Available at: https://pubmed.ncbi.nlm.nih.gov/15013997/ (Accessed: 18 October 2021).

van Doremalen, N. *et al.* (2020) 'Aerosol and Surface Stability of SARS-CoV-2 as Compared with SARS-CoV-1', *The New England Journal of Medicine*, 382(16), pp. 1564–1567. Available at: https://doi.org/10.1056/NEJMc2004973.

Fergie, J. and Srivastava, A. (2021) 'Immunity to SARS-CoV-2: Lessons Learned', *Frontiers in Immunology*, 12. Available at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.654165 (Accessed: 6 December 2022).

Geller, C., Varbanov, M. and Duval, R.E. (2012) 'Human coronaviruses: insights into environmental resistance and its influence on the development of new antiseptic strategies', *Viruses*, 4(11), pp. 3044–3068. Available at: https://doi.org/10.3390/v4113044.

Grifoni, A. *et al.* (2020) 'A Sequence Homology and Bioinformatic Approach Can Predict Candidate Targets for Immune Responses to SARS-CoV-2', *Cell Host & Microbe*, 27(4), pp. 671-680.e2. Available at: https://doi.org/10.1016/j.chom.2020.03.002.

Guarner, J. (2020) 'Three Emerging Coronaviruses in Two Decades', *American Journal of Clinical Pathology*, 153(4), pp. 420–421. Available at: https://doi.org/10.1093/ajcp/aqaa029.

Guo, L. *et al.* (2020) 'Profiling Early Humoral Response to Diagnose Novel Coronavirus Disease (COVID-19)', *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, 71(15), pp. 778–785. Available at: https://doi.org/10.1093/cid/ciaa310.

Human Coronavirus Types | CDC (2021). Available at: https://www.cdc.gov/coronavirus/types.html (Accessed: 18 September 2022).

Jansen, J.M. *et al.* (2019) 'Influenza virus-specific CD4+ and CD8+ T cell-mediated immunity induced by infection and vaccination', *Journal of Clinical Virology: The Official Publication of the Pan American Society for Clinical Virology*, 119, pp. 44–52. Available at: https://doi.org/10.1016/j.jcv.2019.08.009.

Kam, Y.W. *et al.* (2007) 'Antibodies against trimeric S glycoprotein protect hamsters against SARS-CoV challenge despite their capacity to mediate FcgammaRII-dependent entry into B cells in vitro', *Vaccine*, 25(4), pp. 729–740. Available at: https://doi.org/10.1016/j.vaccine.2006.08.011.

Kauffman, K.J., Webber, M.J. and Anderson, D.G. (2016) 'Materials for non-viral intracellular delivery of messenger RNA therapeutics', *Journal of Controlled Release: Official Journal of the Controlled Release Society*, 240, pp. 227–234. Available at: https://doi.org/10.1016/j.jconrel.2015.12.032.

Khoury, M. *et al.* (2020) 'Current status of cell-based therapies for respiratory virus infections: applicability to COVID-19', *The European Respiratory Journal*, 55(6), p. 2000858. Available at: https://doi.org/10.1183/13993003.00858-2020.

Lau, A.C.-W., Yam, L.Y.-C. and So, L.K.-Y. (2004) 'Management of Critically Ill Patients with Severe Acute Respiratory Syndrome (SARS)', *International Journal of Medical Sciences*, 1(1), pp. 1–10. Available at: https://doi.org/10.7150/ijms.1.1.

Li, F. *et al.* (2005) 'Structure of SARS coronavirus spike receptor-binding domain complexed with receptor', *Science (New York, N.Y.)*, 309(5742), pp. 1864–1868. Available at: https://doi.org/10.1126/science.1116480.

Li, F. (2016) 'Structure, Function, and Evolution of Coronavirus Spike Proteins', *Annual review of virology*, 3(1), pp. 237–261. Available at: https://doi.org/10.1146/annurev-virology-110615-042301.

Li, Y.-D. *et al.* (2020) 'Coronavirus vaccine development: from SARS and MERS to COVID-19', *Journal of Biomedical Science*, 27(1), p. 104. Available at: https://doi.org/10.1186/s12929-020-00695-2.

Liu, W. *et al.* (2020) 'Evaluation of Nucleocapsid and Spike Protein-Based Enzyme-Linked Immunosorbent Assays for Detecting Antibodies against SARS-CoV-2', *Journal of Clinical Microbiology*, 58(6), pp. e00461-20. Available at: https://doi.org/10.1128/JCM.00461-20.

Minor, P.D. (2015) 'Live attenuated vaccines: Historical successes and current challenges', *Virology*, 479–480, pp. 379–392. Available at: https://doi.org/10.1016/j.virol.2015.03.032.

Mishra, M., Tiwari, S. and Gomes, A.V. (2017) 'Protein purification and analysis: next generation Western blotting techniques', *Expert review of proteomics*, 14(11), pp. 1037–1053. Available at: https://doi.org/10.1080/14789450.2017.1388167.

Monoclonal antibodies for the S2 subunit of spike of SARS-CoV-1 cross-react with the newly-emerged SARS-CoV-2 (no date). Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7376845/ (Accessed: 18 October 2021).

New insights on the antiviral effects of chloroquine against coronavirus: what to expect for COVID-19? - PubMed (no date). Available at: https://pubmed.ncbi.nlm.nih.gov/32171740/ (Accessed: 26 October 2022).

Newell, K.L. *et al.* (2021) 'Switched and unswitched memory B cells detected during SARS-CoV-2 convalescence correlate with limited symptom duration', *PloS One*, 16(1), p. e0244855. Available at: https://doi.org/10.1371/journal.pone.0244855.

Okba, N.M.A. *et al.* (2020) 'Severe Acute Respiratory Syndrome Coronavirus 2-Specific Antibody Responses in Coronavirus Disease Patients', *Emerging Infectious Diseases*, 26(7), pp. 1478–1488. Available at: https://doi.org/10.3201/eid2607.200841.

Padoan, A. *et al.* (2020) 'Analytical and clinical performances of five immunoassays for the detection of SARS-CoV-2 antibodies in comparison with neutralization activity', *EBioMedicine*, 62, p. 103101. Available at: https://doi.org/10.1016/j.ebiom.2020.103101.

Pal, M. *et al.* (no date) 'Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2): An Update', *Cureus*, 12(3), p. e7423. Available at: https://doi.org/10.7759/cureus.7423.

Park, J.-E. *et al.* (2016) 'Proteolytic processing of Middle East respiratory syndrome coronavirus spikes expands virus tropism', *Proceedings of the National Academy of Sciences*

of the United States of America, 113(43), pp. 12262–12267. Available at: https://doi.org/10.1073/pnas.1608147113.

Pavia, C.S. and Wormser, G.P. (2020) 'COVID-19: Is there a role for Western blots and skin testing for determining immunity and development of a vaccine?', *Diagnostic Microbiology and Infectious Disease*, 98(4), p. 115148. Available at: https://doi.org/10.1016/j.diagmicrobio.2020.115148.

Peiris, J.S.M. *et al.* (2003) 'The severe acute respiratory syndrome', *The New England Journal of Medicine*, 349(25), pp. 2431–2441. Available at: https://doi.org/10.1056/NEJMra032498.

Petrosillo, N. *et al.* (2020) 'COVID-19, SARS and MERS: are they closely related?', *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, 26(6), pp. 729–734. Available at: https://doi.org/10.1016/j.cmi.2020.03.026.

Qian, C. *et al.* (2020) 'Recent Progress on the Versatility of Virus-Like Particles', *Vaccines*, 8(1), p. 139. Available at: https://doi.org/10.3390/vaccines8010139.

Rahman, S. *et al.* (2021) 'Epidemiology, pathogenesis, clinical presentations, diagnosis and treatment of COVID-19: a review of current evidence', *Expert Review of Clinical Pharmacology*, 14(5), pp. 601–621. Available at: https://doi.org/10.1080/17512433.2021.1902303.

Rauch, S. *et al.* (2018) 'New Vaccine Technologies to Combat Outbreak Situations', *Frontiers in Immunology*, 9, p. 1963. Available at: https://doi.org/10.3389/fimmu.2018.01963.

Roy, V. *et al.* (2020) 'SARS-CoV-2-specific ELISA development', *Journal of Immunological Methods*, 484–485, p. 112832. Available at: https://doi.org/10.1016/j.jim.2020.112832.

Salian, V.S. *et al.* (2021) 'COVID-19 Transmission, Current Treatment, and Future Therapeutic Strategies', *Molecular Pharmaceutics*, 18(3), pp. 754–771. Available at: https://doi.org/10.1021/acs.molpharmaceut.0c00608.

Sapkal, G. *et al.* (2020) 'Development of indigenous IgG ELISA for the detection of anti-SARS-CoV-2 IgG', *The Indian Journal of Medical Research*, 151(5), pp. 444–449. Available at: https://doi.org/10.4103/ijmr.IJMR_2232_20.

Adil, M. T., Rahman, R., Whitelaw, D., Jain, V., Al-Taan, O., Rashid, F., Munasinghe, A., & Jambulingam, P. (2021). SARS-CoV-2 and the pandemic of COVID-19. Postgraduate medical journal, 97(1144), 110–116. https://doi.org/10.1136/postgradmedj-2020-138386.

Schwartz, D.A. and Graham, A.L. (2020) 'Potential Maternal and Infant Outcomes from (Wuhan) Coronavirus 2019-nCoV Infecting Pregnant Women: Lessons from SARS, MERS, and Other Human Coronavirus Infections', *Viruses*, 12(2), p. E194. Available at: https://doi.org/10.3390/v12020194.

Sreepadmanabh, M., Sahu, A.K. and Chande, A. (2020) 'COVID-19: Advances in diagnostic tools, treatment strategies, and vaccine development', *Journal of Biosciences*, 45, p. 148.

Umakanthan, S. *et al.* (2020) 'Origin, transmission, diagnosis and management of coronavirus disease 2019 (COVID-19)', *Postgraduate Medical Journal*, 96(1142), pp. 753–758. Available at: https://doi.org/10.1136/postgradmedj-2020-138234.

Vallejo-Illarramendi, A., Marciano, D.K. and Reichardt, L.F. (2013) 'A novel method that improves sensitivity of protein detection in PAGE and Western blot', *Electrophoresis*, 34(8), pp. 1148–1150. Available at: https://doi.org/10.1002/elps.201200534.

Wanyin Tao, X.W. (2021) 'Re-detectable positive SARS-CoV-2 RNA tests in patients who recovered from COVID-19 with intestinal infection', *Protein & Cell*, 12(3), p. 230. Available at: https://doi.org/10.1007/s13238-020-00778-8.

Weiss, S.R. and Leibowitz, J.L. (2011) 'Coronavirus Pathogenesis', *Advances in Virus Research*, 81, pp. 85–164. Available at: https://doi.org/10.1016/B978-0-12-385885-6.00009-2.

*He, F., Deng, Y., & Li, W. (2020). Coronavirus disease 2019: What we know?. Journal of medical virology, 92(7), 719–725. https://doi.org/10.1002/jmv.25766*de Wilde, A.H. *et al.* (2018) 'Host Factors in Coronavirus Replication', *Current Topics in Microbiology and Immunology*, 419, pp. 1–42. Available at: https://doi.org/10.1007/82_2017_25.

Xia, S. *et al.* (2020) 'Inhibition of SARS-CoV-2 (previously 2019-nCoV) infection by a highly potent pan-coronavirus fusion inhibitor targeting its spike protein that harbors a high capacity to mediate membrane fusion', *Cell Research*, 30(4), pp. 343–355. Available at: https://doi.org/10.1038/s41422-020-0305-x.

Xu, X.-W. *et al.* (2020) 'Clinical findings in a group of patients infected with the 2019 novel coronavirus (SARS-Cov-2) outside of Wuhan, China: retrospective case series', *The BMJ*, 368, p. m606. Available at: https://doi.org/10.1136/bmj.m606.

Zhou, P. *et al.* (2020) 'Addendum: A pneumonia outbreak associated with a new coronavirus of probable bat origin', *Nature*, 588(7836), p. E6. Available at: https://doi.org/10.1038/s41586-020-2951-z.