Deanship of Graduate Studies

Al-Quds University

”Identification of immunoreactive COVID-19 epitopes based on M13 phage display library”

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Thesis Approval

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Dedication

I dedicate this work to my lovely family.

To my doctors.

To my colleagues.

To Murad, Suhaib my friends.

To everyone who believed me.

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:

Date: 22/8/2022

Acknowledgment

Pages are not enough to thanks, I would like firstly to take my rights from my hard days, I'm truly thankful to my hard days that build me up and supports me to continue.

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

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Thanks also to Governmental Dura Hospital and central Hebron Ministry of health, both gives me all freedom to collect data and samples hand by hand to face the epidemic.

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Abstract:

Background: COVID-19 is a contagious disease caused by sever acute respiratory syndrome coranavirus2 (SARS-COV2).arise in December, 2019 in Wuhan, China. Highly spreading rate of the virus lead to ongoing COVID-19 pandemic. Coronaviruses (COVs) are positive single stranded RNA (+ssRNA). Name of these sub-groping viruses coming from *coranum* (which mean crown), name are given due to the shape under electron microscope which reflect the appearance of spike glycoprotein on the envelope. , has affected over 2,164,111 people and killed more than 146,198 people in more than 200 countries throughout the world (1). Epitopes are the antigenic determinant part have the ability to recognize human cell receptor, spike protein can recognize Angiotensin converting enzyme 2 (ACE2) on epithelial cell surface and any cells that contain the receptor can be infected with COVID-19, on the same time immunity are produced against epitopes, for example antibodies produced against the virus to neutralize and opsonize COVID-19 virus, and this is what this study about. This study use M13 bacteriophage 12 amino acid peptide library, in which this bacteriophage are alternative for COVID-19 surface, and try to investigate which part of COVID-19 can be identified by human antibodies.

Method: Phage display technique in general contain a library of sequences, these sequences represent peptide variants, study use 12.Amino acid library means that the segments or peptides are expressed on the N-terminal of p III contains a random amino acid sequence for selecting and binding with target molecule. Target molecule used in this study are serum of patient from Palestine, Hebron-west bank. All patient serum are confirmed that contain IGG antibody reactivity against COVID-19 antigen using Ag Rapid Test Device aboot. To increase the probability of antibodies screening and activity against phage library, a pooled serum from 13 patient are made. Before going to main reaction between the library and antibodies in serum, bacteriophage are amplified and enriched inside XLI-blue bacteria, XL1-blue bacteria are grown in LB medium and placed in LB broth then incubated at 37c overnight to reach log phase.5 minute incubation with bacteriophage and both placed inside pre-melted Top agar and pour them off together on previously made LB medium as a double medium. Reactive

phages will be obviously seen. First reaction between phage library and serum of COVID-19 patient occur after taking a copy of amplified phages on Nitrocellulose membrane, membrane are blocked with 5%FCS for 30 minute, pooled serum then are added to react with phage library that coated Nitrocellulose membrane for 2-hours, washing unbounded phages away with PBS-T 3 times, Protein A then added and incubate 1-hour, remove unbounded with PBS-T 3 times, TMB substrate added to observe reaction for 30 minute. Positive clones are picked up for the next reaction, phage are purified by centrifugation at 14,000 for 5 minute, supernatant are taken then 20%PEG with 1/6 volume of 2.5M NACL added to supernatant in fresh tube, incubate overnight at 4c, next day phage are precipitated and appear as a white finger light pellet, precipitate are suspended with 100 micro/liter TBS, then suspension amplified in XL1-blue once again, amplified phage are spotted on NC membrane for the second time(>50 phage are spotted) in a method called Dot-ELISA, process in first reaction are repeated, 4 strong clones(clone1,clone 2,clone 4 and clone 9) are isolated, purified and amplified for third time in XL1-blue bacteria. Each clone (clone1, clone 2, clone 4 and clone 9) are distributed (coat) a 96-well plate, overnight incubation at 4c, all clones are blocked with 5%FCS, addition of 31 patient serum sample (each sample diluted 2 times (1:100 and 1:200)), washing three times, Protein A then added, washing and ABTS substrate are the last.

-all reactive clones from three reactions are taken for DNA amplification, 2 primers Direct2 and Rev2 flanking peptide region and sized about 250bp are used on Thermocycler, PCR product are run on 2% Agarose gel electrophoresis then western blot for fused region on p III. Next generation sequencing illumine for all positive clones and result are aligned and BLAST on NCBI.

Results: alignment showed that there's a massive repeat for DNA sequence insert

(GATTATCATGATCCGAGTCTGCCTACGCTGCGGAAG) in phage 2-s376 for example are repeated more than 10,000 time (appendix). Same result confirmed that sequence (GATTATCATGATCCGAGTCTGCCTACGCTGCGGAAG) enriched in other phages; phage1-s361 repeated 1,483 times, phage 9-s378 repeated 2,083, phage4-s377 repeated 4,573 times (appendix).

This result mean that this sequence are major content of the whole protein, so to ensure that, sequence then are translated to 12 amino acid query sequence, BLAST showed 57% identity in SARS-COV-2 epitopes, 8 identical amino acids are identical in NSP2, ORF1ab polyprotein and ORF1a.

Conclusion: DNA repeat in clones shows strong reaction for local patient serum with peptide variants expressed in it is conformational fold fused with p III on the surface of M13 bacteriophage, positive clones reflect an epitope that may need more to be synthesized to become diagnostic rapid tool for investigation of positive IGG serum for SARS-COV-2 patient.

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Abbreviation used

SARS CoV2	**Sever Acute Respiratory Syndrome Coronavirus 2**
MERS	**Middle East Respiratory Syndrome**
ELISA	**Enzyme-Linked-Immunosorbent Assay**
E. coli	**Escherichia coli**
FCS	**Fetal Calf Serum**
HRP	**Horseradish peroxidase**
IPTG	**Isopropyl-beta-D-thiogalactoside**
PBS	**Phosphate Buffer Saline**
PBS-T	**Phosphate Buffer Saline-Tween 20**
PCR	**Polymerase Chain Reaction**
SDS-PAGE	**Sodium-Dodecylsulfate Polyacrylamide Gel Electrophoresis**
TBST	**Tris-buffered saline and Tween 20**
COVID-19	**Corona virus disease 2019**
DNA	**Deoxyribonucleic Acid**
RNA	**Ribonucleic Acid**
cDNA	**complementary DNA**
RT-PCR	**Real time Polymerase Chain Reaction**
ORF	**Open Reading Frame**
+ssRNA	**positive- single stranded RNA**
CDC	**US Centers for Disease Control and Prevention**
ACE2	**Angiotensin-Converting Enzyme 2**
TMPRSS2	**transmembrane serine protease 2**
NC	**Nitrocellulose Membrane**
POC	**Point Of Care**
CT	**Computed Tomography**

Introduction:

The COVID19 pandemic is considered to be the most crucial global health catastrophe of the century.
COVID-19 Virus can transmit at a high rate, facilitates the spread worldwide, first reported on the 31st of December 2019, in Wuhan, China it become an outbreak emergency, then CDC report COVID-19 in the country.

Coronaviruses (CoVs) are positive-single-stranded RNA (+ssRNA) viruses with a crown-like appearance (*coronam* is the Latin term for crown) because of presence of spike glycoproteins projections on the surface (Cascella, Rajnik et al. 2022).

Coronaviruses epidemics are part of human history, SARS-CoV-1 in 2003, MERS in 2012, and SARS-COV-2 In late 2019.

In 2020 COVID-19 was the third leading cause of death after heart disease and cancer, with approximately 375,000 death reported. First wave of COVID-19 reached a total global cases up to 1 million, this lead that many countries like the UK to enforce the stay-at-home quarantine on March and many other countries on April(Cascella, Rajnik et al. 2022).

Beside the low knowledge of SARS-COV2, RNA viruses have high ability for mutations than DNA viruses, high mutation rate gives more complexity to control identification and cure from pandemic.
The outbreak of COVID19 has had a significant effect on clinical microbiology laboratories over the past few months. A lot of challenges compete within the Covid-19 test. In the pre-analytical stage and or collecting the proper respiratory tract specimen at the right time from the right anatomic site is essential for a prompt and accurate molecular diagnosis of COVID-19 (Wang and Yu 2004).

In this study **"Identification of immunoreactive COVID-19 epitopes based on M13 phage display library"**, we represent the screening of COVID-19 patient serum reactivity against random peptide variants expressed on p III coat protein on the surface of M13 bacteriophage. The newly identified epitopes must react with the high antibody titers seen among positive controls examined by different ELISA kits.

Chapter one: Literature review:

1.1 History:

Coronaviruses epidemics are part of human history, SARS-CoV-1 in 2003, MERS in 2012, and SARS-COV-2 In late 2019. SARS-CoV, MERS-CoV, and SARS-CoV-2, are able to cause severe symptoms and even death, with fatality rates of 10%, 37%, and 5%, respectively(Huang, Yang et al. 2020).

Severe Acute Respiratory Syndrome (SARS) in 2003 emerged in China caused about 800 death and 8400 case, was firs epidemic ever in 21 century(Ortega, Serrano et al. 2020).

The evolution and adaptation of SARS result in a new virulent virus MERS, 2012 outbreak in Saudi Arabia result in many deaths and spread to middle East countries and then worldwide, Most people infected with MERS-CoV developed severe respiratory illness, including fever ,cough, and shortness of breath. Many of them have died(Gastanaduy 2013).

History with coronaviruses are the continued with COVID-19 in late 2019, After the rapid spreading of COVID-19 which was first reported on 31st of December 2019, in Wuhan, China. It becomes officially an outbreak emergency for public health.

CDC report COVID-19 in the country. In 2020 COVID-19 was the third leading cause of death after heart disease and cancer, with approximately 375,000 death reported(Fein 2021).

The first wave of COVID-19 reached total global cases up to 1 million, this lead many countries like the UK to enforce the stay-at-home rule in March and many other countries in April.(Ioannidis 2020)

1.2 Biology

1.2.1 COVID-19 structure:

SARS-COV-2 is a positive single stranded RNA enveloped virus, based on next generation sequencing (NGS) its genome encoding 29,881 bp in length encoding 9860 amino acids(Mousavizadeh and Ghasemi 2021). Gene fragments express four structural protein S, E, M and N where's also express nonstructural proteins like RdRp, nsp2 are encoded in ORF region(Chen, Liu et al. 2020)

RNA fused with Nucleoprotein subunit and connected with inner Membrane protein, all are covered with Envelope protein ending in Spike glycoprotein projections on the surface (Figure 1) (Mousavizadeh and Ghasemi 2021).

1.2.1.1 COVID-19 structural proteins:

Spike protein (S):

The outer most projection on surface, mediate binding to the cell surface receptor angiotensin-converting enzyme 2 (ACE2)(Sofi, Hamid et al. 2020) through the Receptor Binding Domain (RBD) .presence of cellular transmembrane serine protease 2 TMPRSS2 priming virus entry, then virus enter host cell via endosomal/lysosomal system.

Nucelocapsid protein (N):

Most abundant protein it is directly bound to viral RNA, helps in RNA interact with cellular components. N protein also known to decrease immune response via inhibiting type 1 IFN(Lu, Pan et al. 2011).

Enveloped protein (E):

Ion conduction as a viral ion channel (viroporin), Membrane Protein (M) integral membrane protein essential for viral assembly and release.
One of M protein functions is to bind with NFκB and inhibit it lead to low levels of COX-2 result in enhancement of viral pathogen proliferation in host cell(Satarker and Nampoothiri 2020).

Presence of these four proteins are essential for virus assembly and amplification of new virions(Boson, Legros et al. 2021).

SARS-COV-2 Non-Structural Proteins:

Genome encodes 16 non-structural proteins (NSPs) from NSP(0)-(10) and NSP(11)-NSP(16) they all are responsible in replication, transcription, Methylation and other functions, for example: Nsp1 act as host translation inhibitor and degrade host mRNAs, NSP4 viral replication-Transcription complex which helps modify ER(Yadav, Chaudhary et al. 2021).

1.2.1.2 Transmission and life cycle:

SARS-CoV-2 transmit via droplets when an infected person coughs, talks, or exhales in close.(Morawska and Cao 2020),

Spike protein mediate binding to cell receptor (Ace2) through receptor binding domain, after virus entry, virus proteins control host cell metabolism, replicative cycle begins using cell components, ORF1a and ORF1b polyprotein auto proteolytic forming the 16 NSPs yielding a collection called Replicase-Transcriptase-complex (RTC), RTC responsible of early mRNA synthesis. New proteins are formed in endoplasmic reticulum-bound ribosomes, assembly of new virons take place in endoplasmic reticulum-Golgi intermediate compartment (ERGIC) for modification then new virions are released figure 2 illustrate life cycle (Haque, Ashwaq et al. 2020). Newly formed nucelocapsids packaged new RNAs and hydrophobic attract to envelope protein (M proteins) in Golgi apparatus, assembly in a new virions and transported via exocytic pathway out of the cell(Haque, Ashwaq et al. 2020), figure 2 illustrate life cycle:

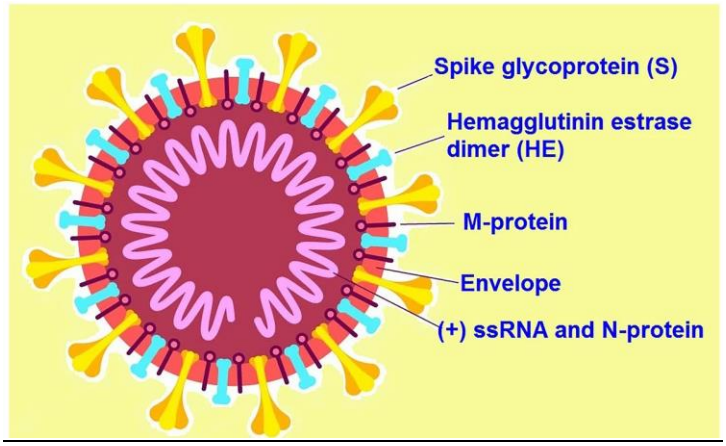


Figure 1: Structure of human coronavirus. (Esakandari, Nabi-Afjadi et al. 2020)

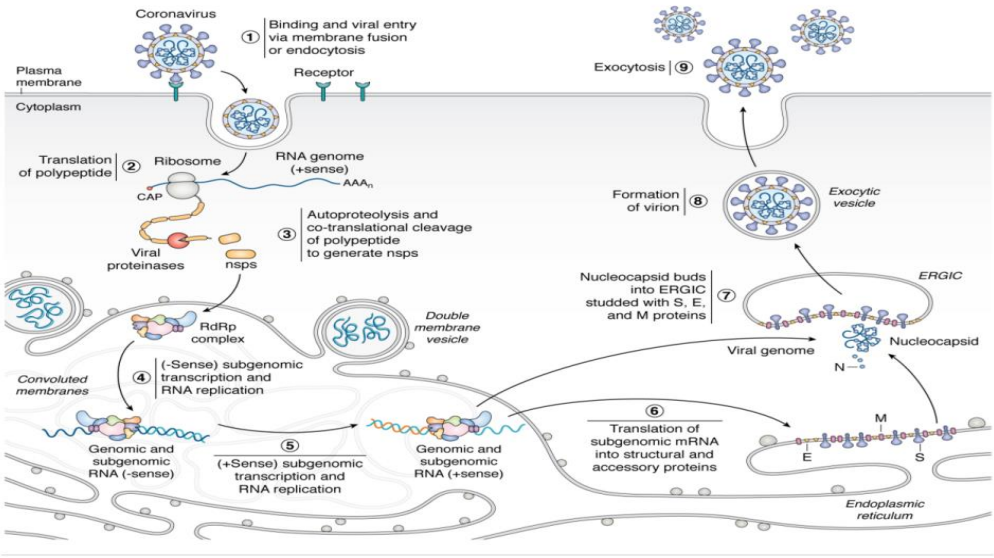


Figure 2: SARS-CoV-2 S spike protein binds to the ACE2 receptor in the epithelial cell of the respiratory tract. The virus undergoes endocytosis, bind to ribosomes in cytoplasm and producing all virus component, then newly virions formed transfer outside the infected cell via exocytosis to infecting more cells (Hartenian, Nandakumar et al. 2020).

1.3 DIAGNOSIS:

Clinical manifestation of SARS-CoV-2 are different among individuals, this variation among local popularity create a challenge to diagnose COVID-19.Diagnosis of COVID-19 depend on many different factors, patient history, clinical state and laboratory tests. For patient with suspected COVID-19, an order of nasopharyngeal, sputum, bronchial aspirate or blood are generally recommended (Rai, Kumar et al. 2021).

Medical condition, history of disease, sign and symptoms are generally work together with laboratory test to prevent any miss-diagnosis with other respiratory system viruses infection. Patient are isolated, chest X-ray, blood sample and nasopharyngeal sample are taken for each patient(Rai, Kumar et al. 2021).

1.3.1 Serological assays:

Serological test also called antibody test is any laboratory procedure investigate sample of blood serum for detecting antibodies or antibody- like substances that may associate with certain disease. Antibodies present in high number while they evoked against a foreign epitope.

Serological methods are many for example: Affinity chromatography, chemiluminescence assay (CLIA), enzyme-linked immunosorbent assay (ELISA), western blotting (WB), immune-fluorescence assay (IFA), and protein microarray.

Antibodies secretion in serum:

Detection of immunoglobulins epically IgM and IgG against COVID-19 surface epitopes dependent on time and method used. Both immunoglobulins are detectable on the first weeks of disease onset, IgM detect recent infection while IGG remains for longer time (Zhou, Xu et al. 2021).

Seroconversion defined as the increasing of secretary antibodies against antigen, within seroconversion, antibodies become detectable in blood. IgM levels increase in first weak after disease onset and reduce on third week, while IGG became detectable after the first week of disease onset and remain high for about 28 week(CHAPEL, HAENEY et al. 2013).

Immunoglobulins secreted from effector B cell (plasma cell) in humoral immunity(CHAPEL, HAENEY et al. 2013). Antigen presenting cells move to lymph-node and present COVID-19 epitope for T helper cell, T helper activate B cell result in secretions of specific IGG antibodies against COVID-19 epitope.

In chromatography rapid tests, membrane on strip have two lines test line coated with gold nanoparticle-antibody conjugates while control line coated with anti-antibody. Sample are added, transported through mobile phase gold nanoparticle-antibody conjugates bind with antigen if present(Udugama, Kadhiresan et al. 2020). Antigen rapid tests give result within 20-25 minute, and can be done to a large group with lower cost than molecular test. It's good

to mention that rapid tests need low limit of detection. Here a figure 3 illustrate the different diagnostic method and target molecule for detection.

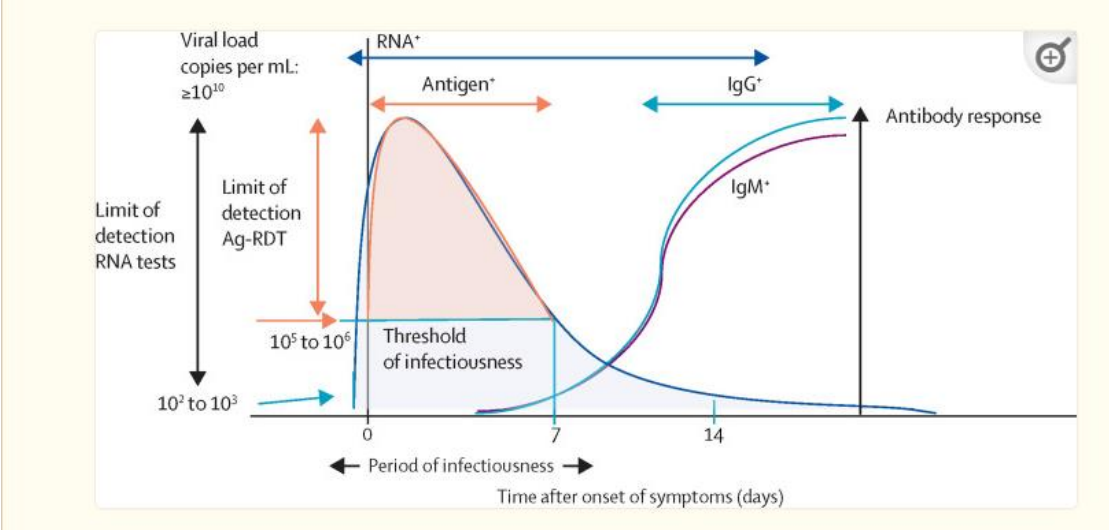


Figure 3: Shows the relationship between time and different diagnostic tests for detection of COVID-19 in one hand and host response in the other hand.Ag-RDT=antigen rapid detection test.(Peeling, Heymann et al. 2021)

1.3.2 Nucleic acid amplification test:

Molecular amplification tests are dependent on understanding genome content and composition for the virus and knowledge of the variation happens within and after pathogen infection. Presence of virus genome in gene bank can facilitate design molecular tools and regents for diagnosis (Udugama, Kadhiresan et al. 2020).

Nucleic acid are the fingerprint gold stander test (Bloomfield, Balm et al. 2015), almost all patient samples are confirmed using nucleic acid tests. Based on many trials and experiments, Saiki et al(Dronina, Samukaite-Bubniene et al. 2021). Develop polymerase chain reaction PCR which become vital diagnostic test in molecular biology, this test depend on ability for multiplication of a specific target DNA using oligonucleotides, presence of proper environment and reagents.

Nucleic acid amplification are set of hybridization test based on designing DNA segment that complementary for target gene of interest. Nucleic acid require a steps like proper

sample collection, DNA/RNA extraction and primer design complementary for gene of interest.

COVID-19 positive single stranded RNA(+ssRNA) contain about 30 thousand nucleotide, and there's a structural important proteins include (nucleocapsid protein (N), matrix protein (M), small envelope protein (E), and surface glycoprotein (S)(Qasem, Shaw et al. 2021), for nucleic acid diagnostic test, use the more conserved gene and low similarity with other coronaviruses give more impact to increase sensitivity while testing.

Corman *et al* using bioinformatical tools analyses and aligned SARS-related virus genome for investigate complementary oligonucleotides, they found 3 regions are able to be amplified and used in molecular investigations: RdRP gene on ORF1ab region.E gene.N gene(Udugama, Kadhiresan et al. 2020).

After that, choosing proper available test based on reagents and conditions take place, because COVID-19 are RNA virus and PCR gives a good impact, RT-PCR are good choice.

COVID-19 RNA firstly extracted from sample, and added to master mix contains double distillated water, forward and reverse primers, a fluorophore-quencher probe, and a reaction mix (consisting of reverse transcriptase, polymerase, magnesium, nucleotides, and additives)(Panel 2021). Then using Reverse transcriptase convert RNA into cDNA using oligonucleotides inside Thermocycler which contain a label signal (probe). System count probe signals and draw flow chart for viral presence (viral load) which give in the end a number of cycles per sample called cycle titer (CT) or cycle titer(Rabaan, Tirupathi et al. 2021).

1.3.3 Computed tomography:

The chest computed tomography (CT) one of most important techniques for respiratory-related illnesses. Previously CT was widely used for SARS and MERS lung abnormalities(Hui, Memish et al. 2014).

Chest x-ray are non-invasive test and can used as clinical diagnosis test for COVID-19 (Yang and Yan 2020).Important feature in COVID-19 patient include bilateral and ground glass opacities, consolidations of the lung are also important for radiologist in COVID-19 infection(Bernheim, Mei et al. 2020).

1.3.4 Limitations:

Serological tests have critical advantages than molecular test in COVID-19 virus diagnosis. Time required for testing are top interest, low probability of contamination within PCR. Reducing sample to result time (SRT) can control transmission of COVID-19(Teymouri, Mollazadeh et al. 2021), Figure 4 illustrate different time period for best identification test compared with time of detection. **Serological tests limitation:**

Window period (undetectable antibodies).

Variable specificity and sensitivity among community.

Cross reactivity among non-specific antibodies (false positive).

Molecular tests limitations:

Undetectable RNA after 2 week.

Take longer time than serological test.

False negative.

Expensive.

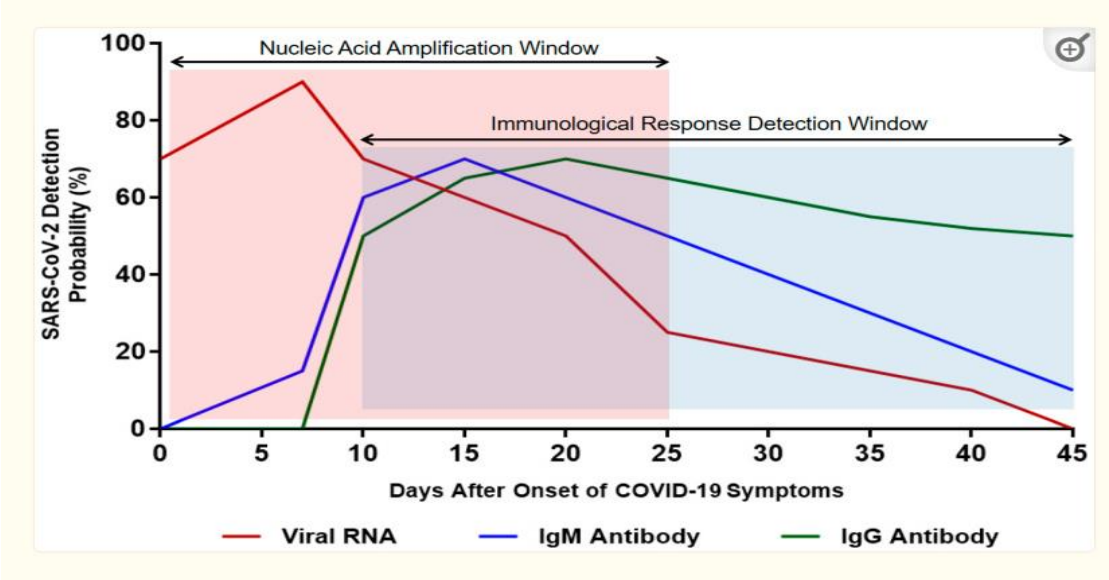


Figure 4: Time detection of SARS-COV-2 using different diagnosis method, Nucleic acid amplification test (NAAT) and serological tests, meanwhile each target of detection are mentioned: Probability's: target (RNA, IgM and IgG) (Qasem, Shaw et al. 2021).

1.4 Treatment:

For COVID-19 self-care are highly critical for minimize disease spreading, there's a many things can help here, like prevention, in which suspected patient should keep at least a 1-metre distance while contact with others, going to health providers centers as soon as possible, tells any one you contact with within previous period, not going to work and stay home, wear a medical mask if it is necessary to go out.

If quarantine and self-care didn't fell patient comfortable, maybe panic lead to another level of admission in hospital, many therapeutic strategies available and approved.

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

Preferred Therapies(Forchette, Sebastian et al. 2021):

Remdesivir: antiviral drug inhibit viral RNA-dependent RNA polymerase (RdRp), U.S. FDA approved for EUA in adults and pediatric patients with severe symptoms on October 20, 2020. (Mouffak, Shubbar et al. 2021

Molnupiravir: RdRp mutagenesis. Phase II/III clinical trials, approved by MerckSharp & Dohme Corp.

Plitidepsin: Targeting eEF1A to inhibit ribosomal activity of host cells, so virus can't replicate. Phase III clinical trial, conducted by PharmaMar, Inc.

Zotatifin: Targeting eEF4A to inhibit ribosomal activity of host cells, so virus can't replicate.

Ivermectin: reduced viral particle proliferation. Phase II. FDA approved.

Monoclonal antibodies:

REGN-COV2: mAb that targets non-overlapping epitopes in the RBD of the S protein, preventing viral entrance. U.S. FDA approved for EUA on November 21, 2020, to treat mild to moderate patients

Bamlanivimab: mAb that targets overlapping epitopes in the RBD of S protein, preventing viral entrance. U.S. FDA approved for EUA in November 2020, revoked as single drug use in April 2021, still in EUA in combination therapies.

Etesevimab: mAb that targets overlapping epitopes in the RBD of S protein, preventing viral entrance. U.S. FDA approved for EUA for combinational therapy of bamlanivimab and etesevimab on February 9, 2021, for mild to moderate patients.

Tocilizumab: humanized monoclonal antibody that binds to interleukin 6 (IL-6) receptor which responsible to inflammation. Approved by FDA.

Dexamethasone: Dexamethasone belongs to the Corticosteroids family, specifically; it is a glucocorticoid, Dexamethasone has both anti-inflammatory and immunomodulatory effect.

In case of Hyper inflammatory phase III: Viral infection become systematic, severe known as sepsis or cytokines storm or even reach multiorgan failure. **There's markers like Decrease** T-cell count, Increase: CRP, interleukins and TNF.

1.5 Prevention:

COVID-19 transmitted via exposure to respiratory droplet, when non- infected person inhale droplet or particulate that contain virus or via touch. For that reason there's general prevention method: always be positive and do not panic, try not to stay in closed areas, keep hygiene to reduce the risk of infection.

Another safer level of prevention: Vaccination and Prophlyxis monoclonal antibodies recommended as : Tixagevimab 300 mg + cilgavimab 300 mg (Evusheld) administered as 2 consecutive 3 mL intramuscular injections (BIII) as SARS-CoV-2 pre-exposure prophylaxis (PrEP) for adults and adolescents (aged ≥12 years and weighing ≥40 kg) who do not have

SARS-CoV-2 infection, who have not been recently exposed to an individual with SARS-CoV-2 infection(Mouffak, Shubbar et al. 2021).

1.6 Host response against viruses:

1.3.1 Viral infection clinical phases:

COVID-19 stages are classified to three phases

Binding: when COVID-19 surface epitopes are complementary for certain target cell. Based on binding affinity Spike protein recognize Angiotensin converting enzyme 2 receptor, then The type 2 transmembrane serine protease (TMPRSS2) on host cell prime viral entry. Both ACE2 and TMPRSS2 are present on alveolar epithelial type 2 cells(Wiersinga, Rhodes et al. 2020).

Cells present epitope on surface at MHC1 and secret interferons to alert immune cells(Wiersinga, Rhodes et al. 2020).

Invasion of upper respiratory tract: Virus migrate through conduction airways to upper respiratory tract. Infecting host begins to show signs like fever and dry cough. Macrophage and Natural killer innate cells begins to react with chemokines secreted by infecting cells result in recruitment to kills infected cells, at the same time dendritic cell take presented epitope to lymph node(Parasher 2021).

Viral sepsis: severe phase of infection, COVID-19 consume coagulation factors, diffuse alveolar damage and thrombotic complications may developed. Patient shows severe symptoms like breath shortening because of immune cells requirement and cytokines storm. Neutrophils, CD4 helper T cells and CD8 cytotoxic T cells are involved(Parasher 2021).

1.7 Immunity against COVID-19:

Physical barriers: the outmost tissue walls that may prevent viruses from invade by using defensins which in result alarming neighboring cells from the danger that will infect cells (Klotman and Chang 2006).The end result is to block invasion and hide receptors via inhibition of gene expression and release of RNAase to cytosol.

Innate immunity: innate immunity begin after the virus escape from physical barriers and infect host cell, cell represent virus epitopes.

This presentation result in: activation of NK while scanning of normal MHC 1 (major histocompatibility complex type 1) on the surface of cells. Activation of Macrophage, when macrophage notice abnormal MHC 1 presentation like a COVID-19 epitopes immediately begin phagocytosis and presenting this foreign epitope for other cells like dendritic cell. Macrophage cells and dendritic cell with all cytokines that they release, also play a key role in activation of adaptive immunity which need 1-2 week to be activated after presentation of epitope in lymph node(Getz 2005).

Whatever the consequences, adaptive immunity begins wide attack against COVID-19 visions and virus infected cells using humoral immunity. Adaptive humoral immunity cause an iso-switching in the secreted antibodies from non-specific antibodies to specific antibodies, these antibodies make: Blocking of extracellular virions. Neutralizing of virions to prevent it from invade new host cells. Opsonization. And Complement activation.

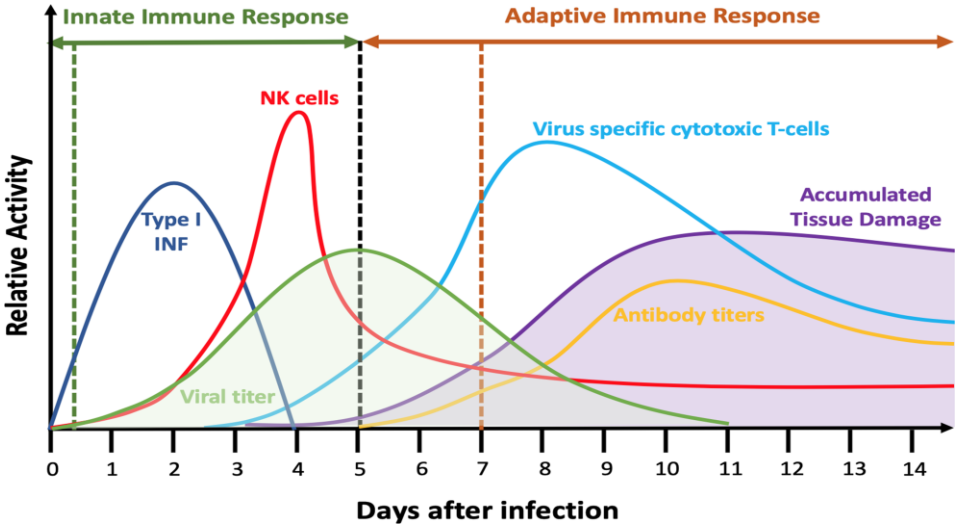


Figure 5: Describe the activation of innate immune response directly after disease onset using type1 INF and NK, then figure shows the transform to adaptive within 5 days, all this area bounded with time, viral titer, antibody titer and T cytotoxic(Sego, Aponte-Serrano et al. 2020).

This study takes this part of immunity (Antibody defense) while secreted in patient with COVID-19 infection and react it with phage library expressed segments as an alternative of COVID-19 surface.

Increase in virus specific IgM in acute phase followed by secretion of virus specific IGG has been observed in COVID-19 infection. IGGs differ in their recognition on epitopes, avidity also should be observed, using target sequence on 96-well this research check specificity of antibodies in binding with phage library expressed segments.

In COVID-19 patient IgM and IGG are continue to increase in the beginning until the third week after onset of disease, then IgM start to decrease, but IGG continue to increase, and still present in plasma after three months of complete recovery. Some studies (Lagunas-Rangel and Chávez-Valencia 2021)reported that in the first two weeks after symptom onset, patients with severe symptoms have more IGG levels than moderate symptoms patients Indeed, viral load on respiratory tissue in severe cases are high which in result, as suspect tissues signals to immune system encourage B cells to produce more quantity than patient with moderate symptoms(Lagunas-Rangel and Chávez-Valencia 2021).

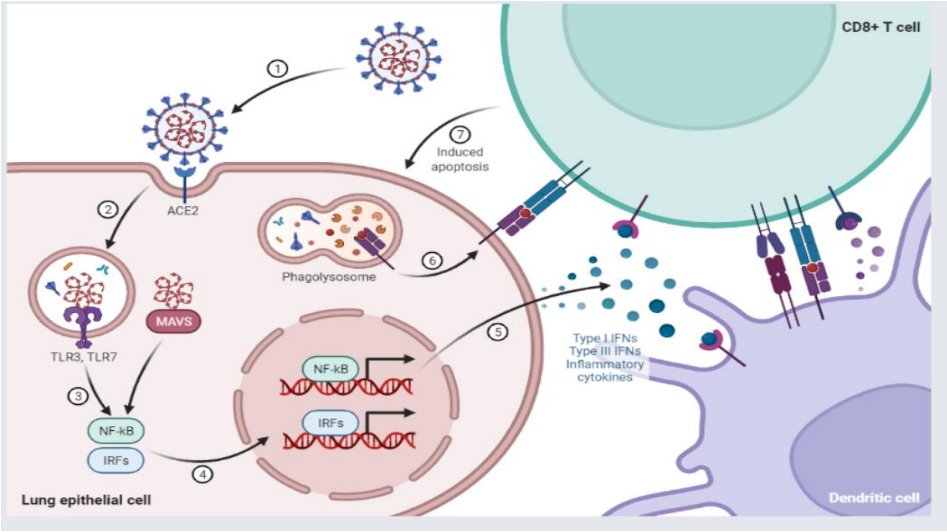


Figure 6: After Coronavirus infect human lung epithelium via the receptor ACE2. A cytoplasmic receptor/sensor bind with it, TLR3/7 and MAVS for example. These receptors begins the inflammation process via activation Interferon Regulatory Factors (IRFs) and NFkB to induce inflammatory cytokines, including interferons (IFN). Dendritic cells (DCs) which in results present the part of virus (sample) to lymph node MHC2 then activate humoral immunity(Santos, Ribeiro et al. 2021).

1.7.1 Cytokines storm:

Simply cytokines storm is the cascade of immunological pathways that's lead for hyper-immunity in response to infection, this storm can take the patient in serious outcomes which may lead to death.

In COVID-19 infection, cells secret signals to immune system as an alert to report that there's foreign antigen, for example: Interleukin-1 (IL-1) family cytokines are key signaling molecules in both innate and adaptive immune systems, both improve body response against wide foreign pathogens, signal starts when an agonist bind to compatible receptor. Arising of COVID-19 severe cases take look back for cytokines circumstances, this circumstances came from immune hyperactivity which cause a damage for organs more than what virus may do, some of these cytokines released by immune cells; IFN-alpha, IFN-gamma, IL-1, IL-1beta, IL-2, IL-2R, IL-6, IL-8, IL-10, IL-12, IL-17D, IL-18, IL- 33, TNF-alpha, TGF-beta, and chemokines such as CCL2, CCL3, CCL5, CXCL8, CXCL9, and CXCL10(Arango Duque and Descoteaux 2014).The point is that many of COVID-19 cases are developed cytokines storm which mostly become fatal.

1.8 Vaccination:

Any prevention process can make difference in COVID-19 pandemic period and waves, if vaccination can prevent or at least decrease consequences of COVID-19 like decline severe cases, hospitalization or even reduce the risk of transmission to other people, so this is a massive success and a good step-forward to eradicate fear of COVID-19(Štefan, Dlouhý et al. 2021).

A good result of effectiveness come from vaccines like BNT162b2, mRNA 1273 and vector vaccines like A2D1222, AD26.COV2.S.(Taking mRNA new vaccines as an example, Ready mRNA are injected intramuscular (IM) when reach cells, mRNA taken to ribosomes and translated into amino acids then folded as complete protein. mRNA then degraded (short half-life). mRNA vaccine produced to encodes to COVID-19 viral spike protein, presented on MHC1 on the surface of host cell, macrophage recognize it as foreign, take part of it, transfer to lymph node, activate B-cells which begins to produce specific IgG antibodies that neutralize foreign surfaces. Next infection with same surface are recognized, memory B cell activated and produce fast response(Covid, Team et al. 2021).

1.9 Pathogenesis:

Using animal models simplify pathogenesis and treatment, previous studies in laboratory mice facilitated our understanding of SARS and MERS (Gretebeck and Subbarao 2015).

Same as SARS-COV, SARS-COV2 receptors in initiate the infection is angiotensin-converting enzyme 2 (ACE2) this receptor is expressed in vascular endothelium, respiratory epithelium, alveolar monocyte, and macrophage(Ni, Yang et al. 2020).

Entry of coronaviruses depends on the binding of the viral spike (S) proteins to cellular receptors and S protein priming by host cell proteases(CHAPEL, HAENEY et al. 2013).

Beginning of cell host entry depend on spike protein (S), this part makes the virus more virulent to recognize alveolar cells in the lung, till now in the middle of the third wave many studies ensure that receptor ACE2 is present in tissues like lung type II alveolar cells, enterocytes of the small intestine, arterial and venous endothelial cells, and arterial smooth muscle cells (Cao and Li 2020).

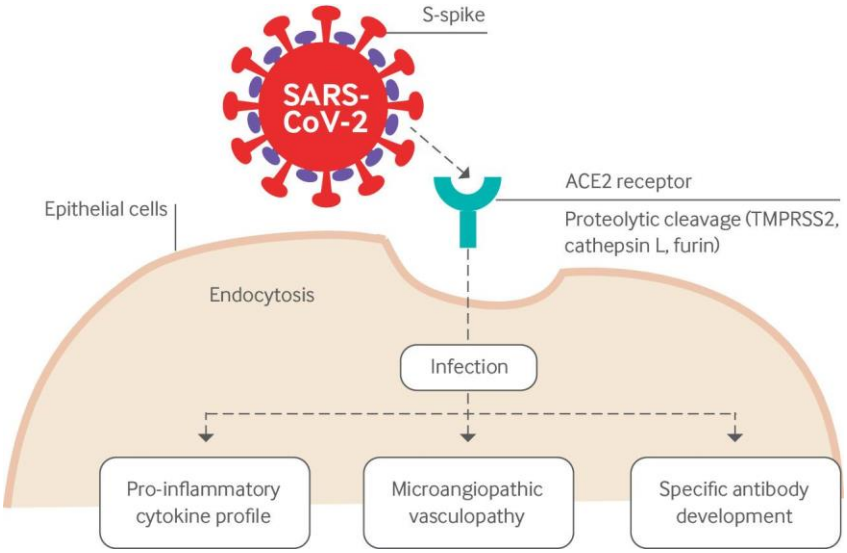


Figure 7: SARS-CoV-2 S spike protein binds to the ACE2 receptor in the epithelial cell of the respiratory tract. The virus undergoes endocytosis, viral maturation, replication, and release of more virus within the cytoplasm infecting the host cell. Consequences of infected cells include pro-inflammatory cytokine secretion, microangiopathic vasculopathy, and B cell secretion of specific SARS-CoV-2 antibodies (Attaway, Scheraga et al. 2021)

1.10 Epitopes:

Epitope is short sequence antigenic determinant part of antigen which bind with target molecule in host cell, but generally epitope is any molecular part in biological system that interact to form a contacting point. However, in cellular system B cell have an epitope to bind with antibody Fab, T-cell also have the famous epitope which represent APC(antigen presenting cell)(Marintcheva 2018).

B-cell epitopes structure can be determined using these method:

- Hydrophilicity: one of first method used to predict B cell epitopes are hydrophilicity, split parts of B cell surface based on a biphasic aqueous/organic solvent mixture, it was only 50% accurate to predict epitopes(Ahmad, Eweida et al. 2016).
- X-ray crystalyzation.
- Antigenic index and other methods based on statistical score.
- Advanced method: Geysen system, simply it is a full automated immunochemical system assay contain a huge library of peptide, analyze overlapping of the sample with the library and count the result, then compare with epitope database (El-Manzalawy and Honavar 2010).
- Experimental studies on COVID-19 surface level helps to understand in one hand how virus can invade, interact and kill host cells and in the other hand clarify how antibody and immune system react with epitopes which improve knowledge in field of therapeutic drugs and vaccinology.

1.10.1 Spike protein epitope (S2): work on cell entry.

Spike protein SARS-CoV-2 S protein is widely conserved in all human coronaviruses (HCoVs) and is involved in receptor recognition, viral binding, and host cell entry. It is one of the most important candidates for the COVID-19 vaccine and clinical research due to its essential functions. Spike protein (S1): work on attachment with ACE2.

With all these functions beside to nuclocapsid epitope, nonstructural protein 13 and other known epitopes. Without these functions of epitopes virus loose its virulence (Jackson, 2022).

Here in this study, pre-made random peptide library, dodecapeptide (Ph.D.-12) library are a random consensus peptide binding sequences against a variety of proteins like cell-surface receptors and monoclonal antibodies.

1.11 Random Phage Display Libraries Technology (RPDL):

Phage Display libraries is a technology for the identification of epitopes or proteins that Mimic protein or non-protein epitopes and protein discovery. Was first developed by G. Smith in 1985, as a method of presenting polypeptides on the surface of lysogenic filamentous bacteriophages. In the phage display technique, a gene encoding a protein of interest is inserted into a phage coat protein gene in a part called p III, causing the phage to display the protein on the outside. And containing the gene for the protein inside, resulting in a connection between genotype and phenotype (Marintcheva 2018).

It is a powerful tool that M13 bacteriophage can be human hand in the experiment, the technician can also replicate it and control the way that he wants to display on the M13 surface (Smith and Petrenko 1997, Yu, Yu et al. 2009)

These displaying phages can be screened for other proteins, peptides, or DNA sequences, to detect the interaction between the displayed protein and those other molecules. So, it has become one of the most powerful and widely used laboratory techniques for the study of protein-protein, protein-peptide, and protein-DNA interactions (Alfaleh, Alsaab et al. 2020).

Bio-panning: Binding between the bacteriophage and receptor sequence in vitro using panning selection technology: incubating a library of phage-displayed peptides on a plate (or bead) coated with the target sequences (Marintcheva 2018).

In simple words, panning is the tool used to fusion between the gene of interest and pre-coated adverse receptors like the key and lock theory (Parmley and Smith 1988).

Selecting of specific Abs by binding with certain library called “panning,” and, in principle, involves the selection of Abs on the basis of their affinity. Isolation of a selected Ab generally involves multiple rounds of panning, every round result in enrichment of elected antibodies. Each round of Ab selection can be divided into panning, removal of unbounded phage, and the elution and amplification of bounded phage Abs for the next round. This step is essential because you cannot complete the rest without having the affinity of selection between library and target (Cwirla, Peters et al. 1990), (Devlin, Panganiban et al. 1990), (Rhyner, Kodzius et al. 2002).

In this study; the Ph.D. TM system or Ph.D-12 Random Peptide Phage Library (Schildkraut 2013) was used; this system based on a simple M13 bacteriophage vector that expresses 12 random folded amino acids. M13 phage libraries 12 commercially available, which present

peptides at the N-terminus of the phage P III coat protein (these peptides displayed at one end of the filamentous phage in 3-5 copies. (Ju and Sun 2017) One phage display technique application is cloning of DNA encoding ligand-binding domains of prokaryotic receptors from chromosomal DNA (Imai, Mukai et al. 2008). This technique in identification of receptor gene needs for probes, so it can be used for cloning prokaryotic receptor genes without prior knowledge of the receptor(Jacobsson and Frykberg 1996). Phage display technique is used to obtain a macromolecule protein (peptides) that mimic the structure of an epitopes. These macromolecule proteins are named mimotopes(Wang and Yu 2004).

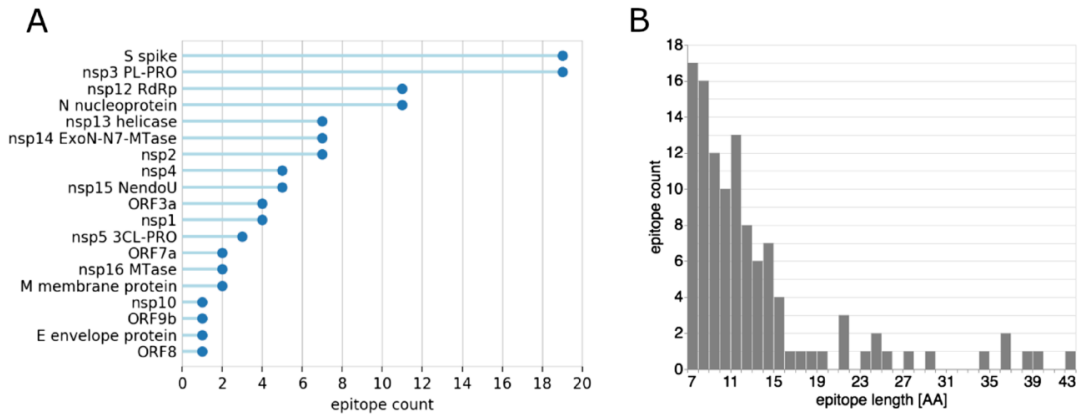


Figure 8**:** Linear epitope BepiPred2 predictions of COVID-19: (**A**) summary count per protein with at least one predicted epitope; (**B**) length distribution. (Phan, Subramanian et al. 2021)

1.12 Study Importance:

The research could help in finding specific 12 amino acid epitopes specific for Covid-19 that are suitable to be used in an immunological assay. Having a specific Covid-19 immunoassay will reduce the need for other expensive and time-consuming molecular tests such as RT-PCR. Also, immunoassay tests can be used in many simple laboratory settings and do not need special types of equipment such as real-time PCR machines.

Having a simple sensitive test for Covid-19 will be much helpful in decision making for any disease control plan, and to reduce the patient's quarantine time or avoid it in case of negative patients.

In future studies, COVID-19 sequencing may give a new level for studying the epitopes using computational methods with the help of bioinformatics. Combined with extensive

mining of sequence databases and structural data, could predict whether a specific protein is suitable for serodiagnosis.

1.13 Epidemiology:

Coronaviruses epidemics are part of human history, SARS-CoV-1 in 2003, MERS in 2012, and SARS-COV-2 In late 2019. SARS-CoV, MERS-CoV, and SARS-CoV-2, are able to cause severe symptoms and even death, with fatality rates of 10%, 37%, and 5%, respectively(Huang, Yang et al. 2020).

Severe Acute Respiratory Syndrome (SARS) in 2003 emerged in China caused about 800 death and 8400 case, was first epidemic ever in 21 century(Satija and Lal 2007).

The evolution and adaptation of SARS result in a new virulent virus MERS, 2012 outbreak in Saudi Arabia result in many deaths and spread to middle East countries and then worldwide, Most people infected with MERS-CoV developed severe respiratory illness, including fever ,cough, and shortness of breath. Many of them have died (Coronavirus 2013).

History with coronaviruses are the continued with COVID-19 in late 2019,

Since arising of COVID-19 Dec 2019 and the high spreading of the virus cross the world, all countries live in panic and riskiness of any new consequences of the virus. Uncontrollable RNA viruses have a long history with unstable events, SARS-Cov and MERS-Cov also cause a highly frequent disease also previously, back to the beginnings of the outbreak until October 1, 2020 the numbers of confirmed cases and confirmed deaths reported to WHO were 33,842,281 and 1,010,634 respectively(Tsang, Chan et al. 2021).

Let's take Palestine as an example of the COVID-19 epidemiology flow, here on March 2020 and before the lockdown, 7 tourist diagnosed as COVID-19 positive, this result of course mean nothing because of the route of transmission, which mean the lockdown done for tourist are ineffective and the virus already transmit for who got close to these tourist, emergency state next month applied by the government. All laboratory staff are alarmed to work in full-shift/24. In Hebron and a photos will show how workflow done to test at least 1000 sample/day in around 12 hour, numbers are crazy and panic of death lead everybody.

4 months later, government because of irresponsible community and high cases number with moderate death cases, reopen partially the facilities and high schools. With all restrictions, ministry of health force wearing masks and take vaccine for everybody who want to take services in government era.

Along with that period, COVID-19 keep growing, more aggressive and many more cases still showed up. On May 2020, South Africa was earliest sample documented, this new mutation got in mind many alarming points: is the new mutation more easily to transfer? Do vaccines affect? What is the location of mutation and how we can test it?

WHO Label	Pango Lineage	Date of Designation		
Alpha	B.1.1.7 and Q lineages	**VOC**: December 29, 2020		**VBM**: September 21, 2021
Beta	B.1.351 and descendent lineages	**VOC**: December 29, 2020		**VBM**: September 21, 2021
Gamma	P.1and descendent lineages	**VOC**: December 29, 2020		**VBM**: September 21, 2021
Delta	B.1.617.2 and AY lineages	**VOC:** June 15, 2021		**VBM:** April 14, 2022
Epsilon	B.1.427 B.1.429	**VOC**: March 19, 2021	**VOI**: February 26, 2021 **VOI**: June 29, 2021	**VBM**: September 21, 2021
Eta	B.1.525		**VOI**: February 26, 2021	**VBM**: September 21, 2021
Iota	B.1.526		**VOI**: February 26, 2021	**VBM**: September 21, 2021
Kappa	B.1.617.1		**VOI**: May 7, 2021	**VBM**: September 21, 2021
N/A	B.1.617.3		**VOI**: May 7, 2021	**VBM**: September 21, 2021
Zeta	P.2		**VOI**: February 26, 2021	**VBM**: September 21, 2021
Mu	B.1.621, B.1.621.1			**VBM**: September 21, 2021

Table 2: COVID-19 mutations(Control and Prevention 2021).

Site of mutations:

ALPHA (B.1.1.7): N501Y, D614G and P681H. All these mutation increase the virulence effect of the virus(Harvey, Carabelli et al. 2021).

Beta (B.1.351): N501Y, D614G, P681H and K417N mutations. K417N mutation will be discussed.

Gamma (P.1): N501Y, D614G, P681H, K417N and H655Y mutations.

Delta (B.1.617.2): E484K, N501Y, D614G mutations. It also contains a K417T mutation and H655Y(Harvey, Carabelli et al. 2021).

Omicron (B.1.1.529): N501Y, D614G, K417N and T478K mutations.

Epsilon: (B.1.427) Epsilon: (B.1.429).

Eta: (B.1.525).

Lota :(B.1.526).

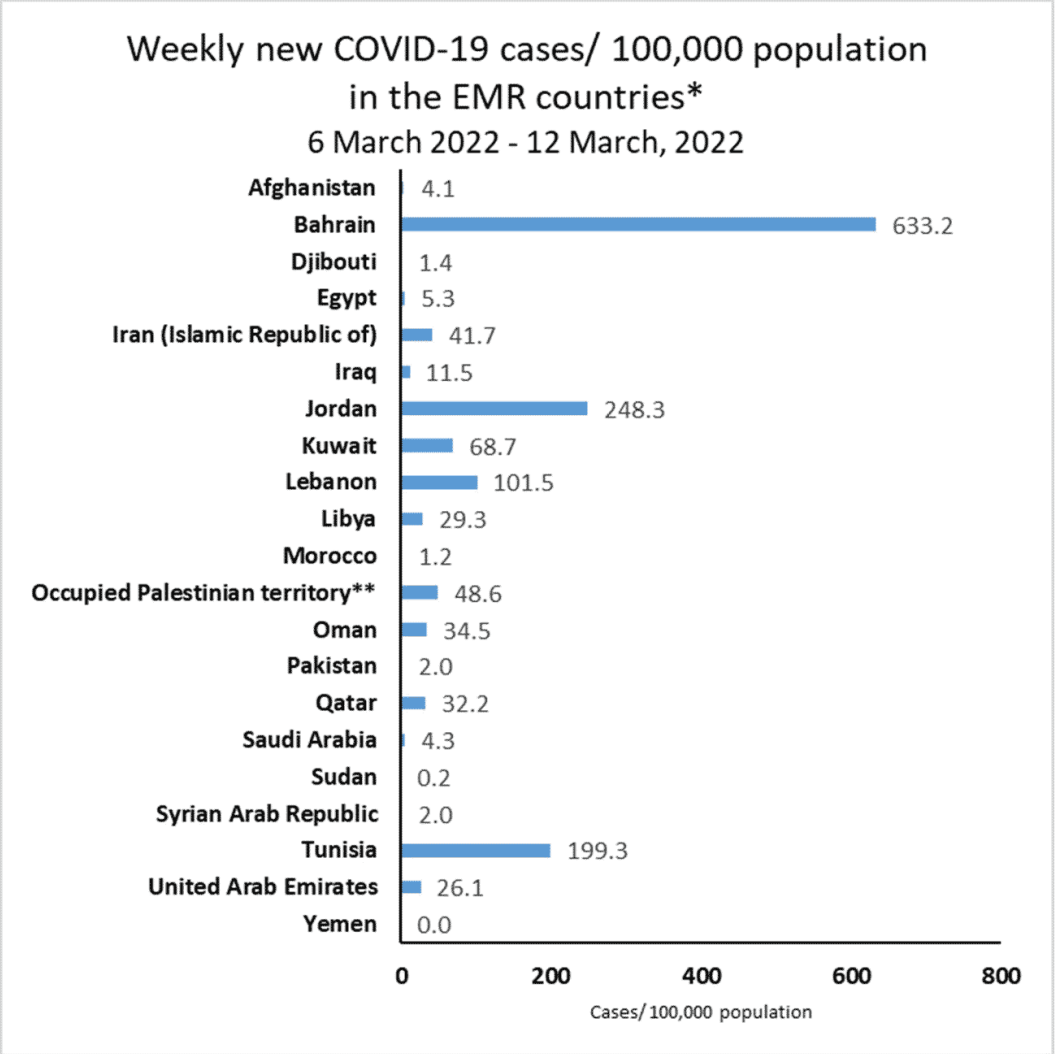
Kappa: (B.1.617.1).

Zeta: (P.2).

Mu: (B.1.621, B.1.162.1)(Control and Prevention 2021).

For almost all mutations, COVID-19 become more aggressive and more adapted with treatment collapse. For example K417N are a dramatic mutation which decline effectiveness of AstraZeneca vaccine in result decline Monoclonal antibody activity against COVID-19.

Even though all governmental restrictions and lockdowns, COVID-19 cause new infections, here a figure illustrate the weekly COVID-19 new cases in Eastern Mediterranean countries (EMR) in a short period Figure 9 showed new COVID-19/100,000:



* The notification rate of newly reported COVID-19 cases is based on data collected by WHO and is affected by the national testing strategy, the laboratory capacity and the effectiveness of surveillance systems. Interpreting the epidemiological situation regarding COVID-19 should therefore not be based on these rates alone
** including East Jerusalem

FIGURE 9: Weekly new COVID-19 cases/100,000 population in Eastern Mediterranean countries (int 2020).

Studies among epidemic around the world prove that elderly people and those with chronic disease are at high risk to develop severe health consequences after being infected with COVID-19. Host factors like age, immunity, use of medications, vaccination and genetic materials. All these factors determines of COVID-19 infection and outcomes (need more data and studies to confirm host factors outcomes in corona). Meta-analysis done on July 2021 consist up to 49,562 patient with COVID-19 across 19 countries and the study report 13 specific genetic variations (genome wide)significant loci that associated with COVID-19(2, leader et al. 2021).

Study on 49.562 shows find four of the loci have similar odds ratios, one of locus is ABO locus (rs912805253) in result this locus suggest that these patients (49.562) are more susceptible to COVID-19. This result define of finding a different locus or different host factors in which virus can invade cells(2, leader et al. 2021). Meta-analysis study support that (genetically) there's a diversity within population reaction with virus, a more studies meanwhile are studying lifestyle, behavior, previous infections and virus transmission effect on severity of COVID-19 infection.

1.14 Study aim and objectives:

The main study aim is to identify 0 amino acids epitopes that are reactive with human sera raised against Covid-19 viral infection. The main objectives can be summarized into the following points:

1- Identification of M13 phage display epitopes using human Covid-19 positive sera.

2- Amino acid sequence analysis of the identified epitopes and finding their correlation with known actual viral epitopes.

3- Expression and purification of the identified epitopes.

4- The use of the purified epitopes as native or bound to a carrier protein in Covid-19 immunoassay tests.

Chapter two: Method and materials

2.1 Serum collection:

COVID-19 positive serum samples were collected from patient with confirmed COVID-19 virus from Hebron-Dura governmental hospital-Covid-19 section, during December 2021. An official cooperation was established with the Hospitals administrations in order to organize sample collection. The total number of collected samples were 29 sample. The main purpose of the collected serum samples was to have a pool of positive sera to be used in screening of phage library during all the current study.

All COVID-19 positive serum samples were confirmed by Abott Serodiagnosis Screening Rapid Test Device, and this was done at the collection site. Only the samples that gave a positive result were used in the current research. Results were also confirmed by patient's clinical symptoms by the resident physicians in the hospital.

2.2 Media preparation:

In the current research; the Ph.D.TM Phage Display Library Kit # E8110S (New England Biolabs, Ipswich, MA, USA). All the used protocols and reagents were according to the manufacture recommendation with some alterations as indicated below.

- Lauria broth (LB): LB media was prepared by dissolving 10g peptone, 5g yeast extract and 5g NaCl in 1L double distilled water, then autoclaved and stored at 4˚C until used.

- TOP agar: for 250ml: 1.7g yeast extract.2.5g NACL and 2.5 peptone. Double distilled water then was added up to 230ml and the solution was dissolved. After that, 1.8g agar was added, autoclaved and stored at 4˚C until used. Melted in microwave and equilibrated at 50˚C before use.
- Bacteria: XL1-blue host strain was used. 5ml LB Broth was inoculated with *E. coli* XL1-blue bacteria and incubated at 37˚C for overnight with shaking.
- IPTG\ X-gal: 1M IPTG (Isopropyl-Beta-D-thiogalactoside) was prepared (2.3g IPTG was dissolved with 10ml D.D.H2O). X-gal (5-Bromo-4-chloro-3-indolylbeta-D-galactoside) also was prepared (0.2g X-gal was dissolved in 10ml DMSO (Dimethyl sulfoxide). X-gal was stored at -20˚C in the dark.

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

• TBST (Tris-bufferd saline and Tween 20): To 500ml TBST, (25ml Tris 7.5, 1.5ml 5M NaCl, 250 µl Tween-20 and 500ml D.D.H2O.

•Blocking Buffer: 0.1 M NaHCO3 (pH 8.6), 5 mg/ml BSA, 0.02% NaN3.

2.3 Bacterial strain and M13 phage library maintains:

XL1 blue bacterial colony isolated from fresh LB agar plates was inoculated into 15 ml tube containing 5ml LB broth media with 5mg/ml tetracycline, all bacterial strains used in the study are resistant to this antibiotic. (Loss of F-factor in nonselective media is insignificant as long as cultures are not serially diluted, and the stock cultures are kept with tetracycline). This stock culture was kept at 4^{o}C and it was renewed each 2-3 days. Also bacterial (XL1-Blue) was also kept on LB agar plates with tetracycline for longer about 2-3 weeks and it was renewed each time.

2.3.1 Phage titration:

1-In 15ml tube, add 5ml LB broth with 200 µl XL1 blue colonies from previously growing overnight culture.

2- Place tubes on water bath at 37^{o}C and shaking overnight to reach mid log phase.

3- Dissolve top agar and dispense 3 ml on tube (one per expected dilution), then place it on shaker at 45^{o}C.

4-Pre-warm LB media at least one hour before culture at 37^{o}C.

5-In 1.5ml microfuge tube dispense 200 µl of LB with bacteria from step 1 (one per expected dilution).

6- Make a phage dilution by adding for first the 1.5 ml microfuge tube 1 µl of original phage that is taken from the M13 phage library, transfer 5 µl to the next tube, and then do 100-fold serial dilution. At this stage two tubes were left without M13 phage to be used as negative control.

7-After the addition of all components vortex quickly and incubate for 1-5 minute at room temperature to carryout infection between phage and bacterial cells.

8-after incubation transfer cells with phage to tube contains top agar, vortex briefly and immediately pour its content into LB agar plate, gently rotate to spread the content and then leave it to dry for 10 minute.

9-Invert plates and incubate for overnight at 37ºC.
10-Count plaques on plates that have (approximately 100 plaques). Multiply each number by the dilution factor for that plate to get phage titer in plaque forming units (pfu) per 10 µl.

2.4 Plaque lift and screening of reactive phage on nitrocellulose (NC) membranes:

For this purpose, a nitrocellulose membrane-based ELISA for the detection of sera containing COVID-19 IGG antibodies was performed, in which we used the pooled COVID-19 sera to be reacted with the expressed phage peptides followed by reaction with a second antibodies to react with human IGG antibodies (in this case we used Protein-A HRP conjugated that reacts human IGG).

Initially and after phage titration, the ideal phage titer that shows best plaques distribution were chosen and based on this dilution about 20 plates were prepared to have maximum number of separated phages to use in phage reactivity with positive human sera. All newly prepared agar plates with separated phages were kept at 4ºC to let the top agar to dry and not to be adhere to nitrocellulose membranes upon plaque lift. For each LB agar plate that containing separated plaque; a nitrocellulose membrane was placed directly over the plaques for 3 minute to allow transfer of the phage copy into the membrane.
Stabbing around the edges of the nitrocellulose membrane using needle or pencil to organize membrane orientation for future use. The plates then were incubated for 30 min at 37°C and then incubated for 1 hour at 4°C after that membrane removed gently and kept to dry overnight at room temperature inside 1MM Whatman membrane.

NC membrane-based ELISA for the detection of reactive phages with human pooled sera.
A nitrocellulose (NC) membrane was treated as a solid-phase support for the detection of COVID-19 reactive pooled positive serum. NC membrane contain antigen which are located on the surface of M13 phage previously coated with, in this experiment NC were incubated in sealing nylon bags, and then filled with 10 ml blocking buffer (PBS with 5%FCS); this step is needed to prevent non-specific antibody binding to NC membrane and accumulation of colored background. Blocking was done for 30 minute on slow shaker. The blocking step was followed by the addition of 20 µl of pooled positive sera (1:500 dilution) into the sealed nylon sac containing the NC membrane, the membrane was incubated for 2 hours at room temperature with shaking. After that the NC membrane was taken out from the sealing bag and washed three times with PBS-Tween for 5 minute each time, then **immediately** and

without leaving the membrane to dry, the second antibody and in this case we used (Protein A) that was diluted (1:5000) in blocking buffer was added and the membrane was incubated another time at room temperature on a shaker for 1 hour. This was followed by last washing step, another time; three times 5 minute each using PBS-Tween. final the last step was the addition of colored substrate that is first prepared as (30 mg of TMB (Sigma-Aldrich, USA) in 5ml ethanol, from this stock solution 1ml of the TMB was mixed in 19ml citrate buffer (pH 5.2) and 30 µl hydrogen peroxidase (H_2O_2). The substrate solution was left for about 20-30 minutes or until getting clear blue dots without dark background.

2.5 Picking of reactive plaques with human Covid-19 positive sera.

After screening, plaques that react with pooled positive sera were removed (picked) by the aid of sterile micropipette tip. Each positive reacted plaque was picked-up and placed on 1.5 ml microfuge tube containing 300 µl LB broth, vortex and left at room temperature for about 30 minutes and then stored at 4°C until further use.

2.6 M13 bacteriophage purification and amplification (prepare bio-panning second round):

M13 phages containing the reactive peptides were enriched and precipitated for further analysis. This was done first by centrifugation to remove the carry over agar and bacterial cells by centrifugation at 14,000rpm for 5 minutes. Then phages were used to infect bacterial cells as indicated above. In the second day the broth media containing the released M13 phages were centrifuged to remove the bacterial cells and to 1ml of the supernatant 200µl of 20% PEG/2.5M NaCl was added. Phage was allowed to precipitate for overnight at 4˚C. The PEG precipitated phages were then spin at 14,000 rpm at 4˚C for 10 minutes and the supernatant was discarded. The phage pellet was suspended with 0.1 ml TBS solution. The Amplified phage was used in a second M13 phage surface binding/screening, and the amplified phage of the second screening round was used in a third round of screening. The M13 phage that was produced from the third screening round was amplified and named as the final amplified phage. The resulted amplified phage was tittered on LB/IPTG/X-gal plates.

2.7 Dot-blot ELISA screening over picked eluted and precipitated samples:

All phages that showed a reactivity were amplified in XL1-blue bacterial cells and then suspended in TBS (tris-buffered saline), all amplified single phage sample were applied on NC in duplicate (undiluted and then diluted 1:100 on NC membrane. This was done after the NC membrane placed into 1X PBS until become completely wet then placed on dot blotter apparatus (Bio-Rad, USA) which was connected to vacuum pump. Then immediately before the NC become dry, all samples that contain reactive M13 phage are blotted on the membrane (50 µl for each dot), then after adding all samples the vacuum pump turned off and membrane let to dry overnight at room temperature between 1mm Whatman membrane. Next day the membrane that was already coated with the antigen (M13 phage reactive peptides), was first blocked with PBS with %5 FCS for 30 minute with shaking at RT. Then this was followed by the addition of 1:500 diluted pooled positive serum 1:500, and then incubate for 2 hours at RT with shaking. After this reaction the membrane was taken out washed 3 times with washing buffer solution PBS-T for 5 minute each to remove unbound phages and remnant of serum proteins. Protein-A was used as secondary antibody (it was used at a dilution of 1:5000 in blocking buffer), after its addition the NC membrane was incubate 1 hour at RT with shaking. Later the NC membrane was washed 3 times for 5 minute each. After the last washing step TMB colored solution was added and this was done as indicated above (see 2.4).

2.8 96-well ELISA using selected reactive M13 phages

Reactive phages which were diluted on Dot-Blot ELISA are again brings to 96-well for further investigations about reactivity, phages 100 µl are placed in deep well in triplicate, incubated overnight at 4˚C, coated part still and rest are discard in sink, 100 µl blocking buffer in each well, incubate 30 minute at RT, washed 3 times with PBS-T 5 minute each, 100 µl (1:200) pooled serum was added, incubated for 2 hours at RT, then washed 3 times 5 minute each. Then a 100 µl secondary antibody (Protein A-HRP) (diluted 1:5000) was added to each well, incubated for 1 hour at RT and then washed 3 times with PBS-T, 100 µl of ABTS substrate was added and incubated for 30 minute. The color change (to green color) was measured at 420 nm using ELISA auto-reader. In this experiment amplified phages that showed high antibody titer were coated again into 96-well. Four amplified phages were chosen to be tested (1, 2,4 and 9) if there are reactivity between them and sera of **COVID-19** infected individuals.

The below sketch represents the ELISA plate organization and the used diluted individual sera samples.

S1a: sample 1 crude.

S1b: sample 1 (diluted 1:100)

S1c: sample 1 (diluted 1:200)

1a	1b	1c	2a	2b	2c	3a	3b	3c	4a	4b	4c
5a	5b	5c	6a	6b	6c	7a	7b	7c	8a	8b	8c
9a	9b	9c	10a	10b	10c	11a	11b	11c	12a	12b	12c
13a	13b	13c	14a	14b	14c	15a	15b	15c	16a	16b	16c
17a	17b	17c	18a	18b	18c	19a	19b	19c	20a	20b	20c
21a	21b	21c	22a	22b	22c	23a	23b	23c	24a	24b	24c
25a	25b	25c	26a	26b	26c	27a	27b	27c	28a	28b	28c
29a	29b	29c	30a	30b	39c	31a	31b	31	+	b	-

2.9 Polymerase Chain Reaction (PCR).

All selected reactive phages that were detected by phage dot-blot (Dot-ELISA) and by plate ELISA were used to amplify the present insert in M13 phage that represents the 12 amino acid sequence. DNA amplification was done by polymerase chain reaction (PCR). This PCR amplification analysis was done using direct and reverse primers targeting a portion of the M13 bacteriophage gene.

The direct 2 (TTATTCGCAATTCCTTTAGTG) and Rev2 (CCCTCATAGTTAGC GTAACG) primers were used for this purpose. PCR analysis firstly was carried out for 16 sample (including the negative controls), a master mix tube was prepared as followed: 360µl double distilled water, 40µl of 20 pmoles of Direct2 primer and 40 Rev2 primer, then it was mixed well and 22µl was dispensed in each Ready Mix PCR tubes (Syntezza, Jerusalem)(Adwan 2014) PCR TUBE followed by the addition of 3µl from each phage clone. The used thermal profile in thermocycler involving 5 min at 95°C to initial denaturation, followed by 36 cycles each of 30 second at 95°C to complete denaturation, 30 sec at 55°C Tm (depends on the used primers) 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.

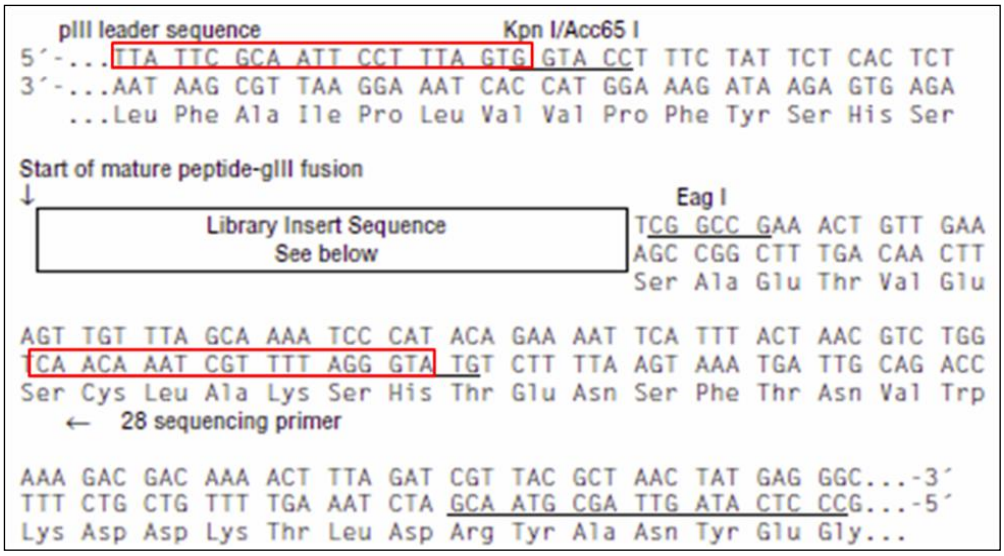


Figure 10: M13 phage sketch showing the insert location and the place of the used primers. (Direct2: at 5'end and Rev2)

2.10 Agarose Gel Electrophoreses:

PCR products were run on a 2% agarose gel (2g agarose, 100ml 1X TAE and 10µl Ethidium bromide in 1X TAE electrophoresis running buffer (242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (Ph8.0)). The Gene Ruler 100bp DNA ladder (Thermo Scientific, # SM0371) was used for sizing PCR amplified products.

2.11 DNA purification:

PCR products were purified by Gene JET PCR purification Kit (Thermo Scentific, USA), according to manufacturer's instructions.

2.12 DNA sequencing:

Positive clones are sequencing using Next Generation sequencing illumine, sequencing by synthesis method, result then are aligned in BLAST with database especially with respiratory viruses.

2.13 Western blotting

Lysed phage products (clone 2) were suspended in PBS and were boiled 3 minutes in SDS-PAGE sample buffer and separated by gel electrophoresis. The proteins were transferred to nitrocellulose membrane (0.45 □m, S&S, Dassel, Germany) and blocked with 5% FCS in PBS for 1 h at RT. Membrane strips were then incubated with anti-covid-19 pooled sera (1:5000 dilution in 5%FCS in PBS) for 2 h at RT. The membranes were washed three times with PBS-T (0.1% Tween 20 in PBS) reacted with HRP-Prot-A (1:5,000 in dilution buffer), for 1 h at RT. After additional washes chromogen substance (DAB) was added.

2.14 Bioinformatics analysis:

Raw Illumina sequencing data was generated from all analyzed PCR amplicons as FASTQ files of read1 (forward) and read2 (reverse) for each individual sample. These sequence reads were uploaded to Galaxy platform at (usegalaxy.org) for further sequence processing and analysis. Initially raw sequences were filtered for quality control at a phred score of 20, followed by merging forward and reverse reads, the amplified specific genes were selected according to their specific sequence length and sequence identity. The selected sequence reads were aligned to identify similar sequences (epitopes) and the common identified sequences were analyzed for sequence homology using BLAST analysis tools.

Chapter three: Results

3.1 Serum samples:

All serum samples that were taken from patient with clinical signs were confirmed cases for COVID-19. This was done using COVID-19 Ag rapid tests device. From these positive confirmed samples a pooled sera was prepared and it was used all over the study. At this stage it was not essential the metadata of these samples as only if they are positive and they have high antibody titers. Also in the coming tests we only concentrated on the presence of anti-COVID-19 IgG antibodies.

3.2 Screening of M13 random phage display library:

The M13 phage library titer indicated by the phage library manufacturer (New England Biolabs, Ipswich, MA, USA), was estimated to be 10^13 plaque forming units (pfu)/ml. It is known that the titer of the phage library kit decreases along storage time, for this reason and for obtaining a workable number of plaques (separated plaques) in each plate; the phage library was titrated using different dilutions of phage library starting from 10^3 down to 10^16. Each dilution was used to infect 200µl of freshly growing XLI-*BLUE in log phase* bacteria followed by plating on LB agar plates that include IPTG as described in materials and methods. After performing phage titration test, it was found that the phage titer was up to 10^11(pfu)/ml.

Based on this experiment a dilution of 10^9(pfu)/ml which showed the best separated phages was chosen to be used in preparation of many agar plates with M13 phages. So, about 20-30 plates were prepared for screening the reactive M13 phages that supposed to contain peptides resemble the COVID-19 epitopes.

3.3 Selection of reactive M13 phages against COVID-19 antibodies:

The final amplified M13 phage was grown on agar plates (about 15 plates) in a dilution of 10^9 (pfu/ml), the obtained plaques from each plate were transferred into NC membrane and then screened to their reactivity against anti-*COVID-19* pooled sera. Plaques that were lift on the NC membrane from each plate were screened by 1:500 diluted anti-*COVID-19* pooled sera as first antibody and 1:5000 diluted protein A-HRP as a second antibody (Figure 8). The highly reactive plaques (plaques that gave strong color signals) were transferred and

incubated in 300µl LB media for further analysis. The total number of the obtained M13 strong reactive phages was 120 plaques from a total of 10 plates that are estimated to have at least 1,000 plaques (assuming in each plate there are only 100 plaques). From the total number of the obtained M13 reactive phages (1000 plaques); 50 reactive plaques were picked up and scan in next panning cycle.

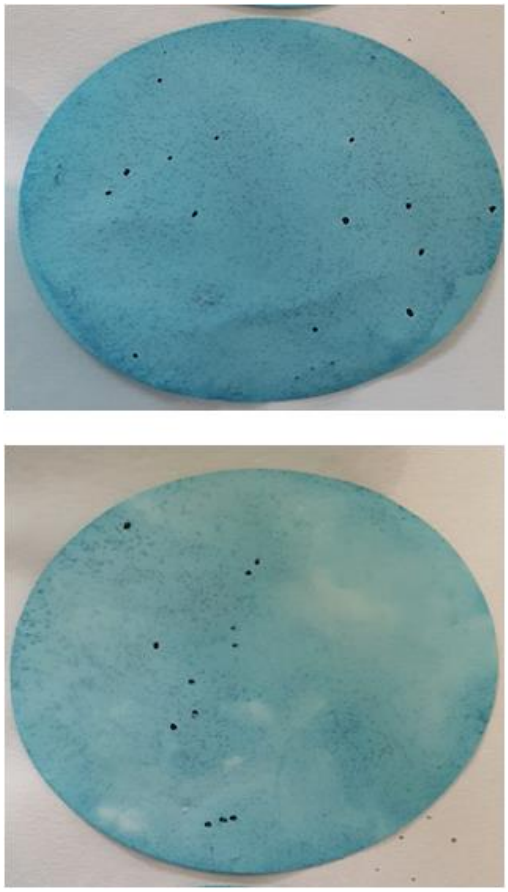


Figure 11: Represented selection of reactive M13 plaques that showed strong interaction with anti-Covid-19 pooled serum. The figure shows two of nitrocellulose membrane contain plaques that were formed after their amplification inside 200 µl l XLI-BLUE bacteria with IPTG, later on plaques with strong dark blue dots were picked up for further analysis.

Table 1: Summary for the number of obtained plaques from 10 different plates and that were reactive with anti-COVID-19 high antibody titer pooled sera.

Plate number	Number of reactive phages	Total
1	**128**	**1098**
2	**163**	
3	**90**	
4	**119**	
5	**95**	
6	**76**	
7	**87**	
8	**55**	
9	**136**	
10	**57**	
11	**42**	
12	**50**	

3.4 Proving reactivity of selected reactive M13 phages by Dot-ELISA (Phage Dot-blot Assay):

The 50 selected phages were amplified individually in 5 ml LB media containing XL1-blue cells and IPTG for peptide induction. After growing of all the 50 clones the produced peptides were precipitated and concentrated in about 0.ml of Tris-Buffered Saline (TBS). About 20 µl of the 1:100 diluted precipitated M13 peptides were spotted on NC membrane with the aid of vacuum suction. The membrane was dried and then used in dot-ELISA to select the optimal reactive clones against pooled human positive COVID-19 sera. Figure 12, shows a representative results of this dot-ELISA.

Figure 12: A representative results of dot-ELISA. Different clones of M13 peptides were spotted on NC membranes and then reacted with pooled anti-Covid19 sera.

Based on this experiment, 10 strong positive clones were selected, these clones were named as clone 1 to clone 10. From these 10 clones the 4 strongest clones were selected: clon1, clone 2, clone 4, and clone 9. And they were used in the coming ELISA assay.

3.5 ELISA assay of M13 reactive clones:

In this assay we tested the reactivity of individual collected positive COVID-19 sera samples against three of the selected above M13 phages (clon1, clone 4, and clone 9). In this assay the extracted peptides included in these M13 phages were used to coat an ELISA 96 well plates. Positive and negative sera were used and beside regular blank ELISA well we coated some wells with M13 clone that proved not to be reactive with human COVID-19 positive samples. In this ELISA assay we only tested the reactivity of IGG antibodies present in positive sera, since the second antibody was Protein-A HRP conjugated. Each of the

individual sera samples were used in three dilutions (1:10, 1:50, and 1:100). The results indicate that, the three selected phage groups used in this ELISA assay; showed consistency and high antibody titer in terms of their reactivity against the examined phages Figure 13:

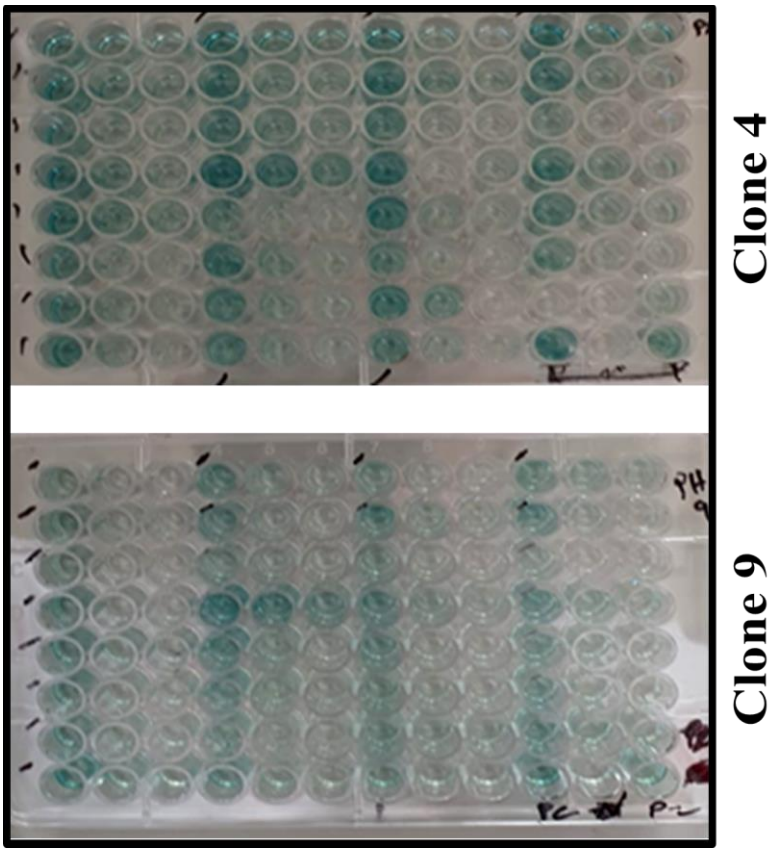


Figure 13: Phage ELISA Binding Assay for different sera of *COVID-19* infected individuals reacted amplified phages. In this figure only the plates of clone 4 and 9 are shown, but similar results were also obtained for clone 1.

3.6 PCR amplification 48bp coding 12 amino acids of the selected M13 phages:

For all the final selected 10 clones and a concentration on the strongest 4 selected clones that were also used in ELISA assay (3 of them), the inserts that transcribing the 12 amino acids were amplified and sequenced. For the insert amplification we used two systems of PCR with the same forward primers but different reverse primers. The main purpose of using two systems was to maximize the insert amplification process. Figure 11 shows agarose gel electrophoresis of the amplified 4 strong selected clones and some other clones that also

showed strong interaction with COVID-19 positive sera in dot blot analysis. The amplified bands were of a uniform size (250 bp) that all were proven to include a different region of 48bp coding for the 12 amino acids epitopes Figure 14:

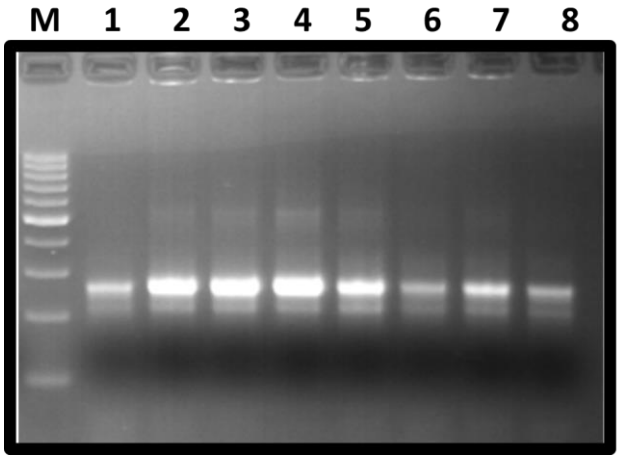


Figure 14: Agarose gel electrophoresis analysis of PCR products targeting the 12 amino acids included in the selected phages. 1: phage 1, 2: phage 2, 3: phage 4, 4: phage 9, 5: phage 5, 6: phage 6, 7: phage 8, 9: phage 10.M: 100bp molecular weight marker.

The amplified PCR products were sequenced using NGS sequence methodology and this to obtain the maximum possible abundant 12 amino acids sequences. It is known that these selected phages could have minor contamination of other epitopes.

3.7 Western blot analysis:

analysis of binding activity of COVID-19 positive IGG human pooled sera against fused selected 12 amino acids expressed on the surface of filamentous phage as N-terminal fusions to p III coat proteins. Result confirm the presence of p III coat protein band that weighted 50 kDa. p III react with IGG since protein A used as secondary antibody.

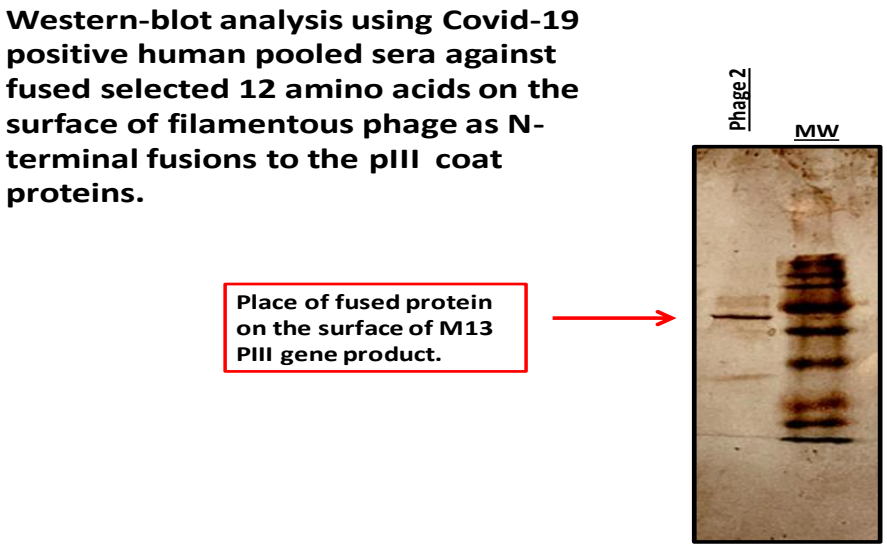


Figure 15: shows result of Western blot analysis, band represent 50 kDa confirm the presence of p III coat protein band that weighted 50 kDa.

3.8 Bioinformatics DNA sequence analysis of the obtained sequences.

Next generation sequencing is advantageous over Sanger DNA sequencing since it gives the sequence for each most DNA PCR amplified segments. So suppose we have one band PCR product then NGS analysis will show the sequence of all DNA molecules which this band is composed from. In contrast DNA Sanger DNA sequencing only gives the sequence of the most abundant amplicon and in many cases it will give (N: unidentified nucleotide) if at this specific position more than one type of nucleotides could occurs. NGS analysis enabled us to see the most abundant insert sequence in M13 phage or the most abundant peptide (12 amino acids) that found in the analyzed M13 phage.

Using Galaxy online platform we developed a workflow that join the reverse and direct sequences of the specific sequenced phage and at the end the workflow could count the frequency of the repeated segments after making an alignment to ensure the presence of the same DNA sequence in the M13 phage insert site.

The major finding in this bioinformatics analysis was represented by identifying one major sequence for the 12 amino acid sequence that was repeated in the most analyzed M13 phages, this sequence was:

(GATTATCATGATCCGAGTCTGCCTACGCTGCGGAAG)

The above sequence was highly repeated in M13 clones that gave strong signals with positive Covid-19 sera, it was repeated 9,576 times in clone 2 of M13 phage, 1,483 times in clone 1 M13 phage, 2,083 times in clone 9 M13 phage, 2,083 times in clone 4 M13 phage (Figure 15). (Detailed results on Appendix).

Translating the above indicated sequence taking into account the correct open reading frame; gives 12-amino acid peptide sequence: **DYHDPSLPTLRK.** Based on protein BLAST analysis this peptide sequence gave 57% identities to nsp2, ORF1a and ORF1ab in SARS-COV2 with10/14 positive amino acid repeat (Figure 16) (appendix).

Comparison alignment with respiratory viruses shows:

3 of 12 identity with query sequence with MERS-CoV (taxid: 1335626) in Nsp12, ORF1ab polyprotein, 1AB polyprotein.

4 of 12 amino acids identity with query sequence with Influenza A virus (taxid: 11320) in HA2, hemagglutinin. 6 of 12 amino acids identity with query sequence with Influenza A virus (taxid: 11320) in non-structural protein 1. Figure 16

Virus name/insert	Name of epitopes	Identity	QUERY Sequence: DYHDPSLPTLRK
MERS-CoV (taxid:1335626)	**Nsp12,ORF1ab polyprotein, 1AB polyprotein**	(%75)3/4	LPTR LPT+
SARS coronavirus (taxid:694009)	-	-	- -
Influenza A virus (taxid:11320)	**HA2, hemagglutinin nonstructural protein 1**	(%100)4/4 (%60)6/10	DYHD- -------- DYHD- ------- - -HD----PSLP - -HD -----PSLP
SARS-CoV-2 (taxid:2697049)	**Nsp2, ORF1a polyprotein, ORF1ab polyprotein**	(%57)8/14	DYHD PS- LPTLRK +YH + +S L +TLRK

Figure 16: shows amino acid alignment with respiratory viruses using BLASTp.

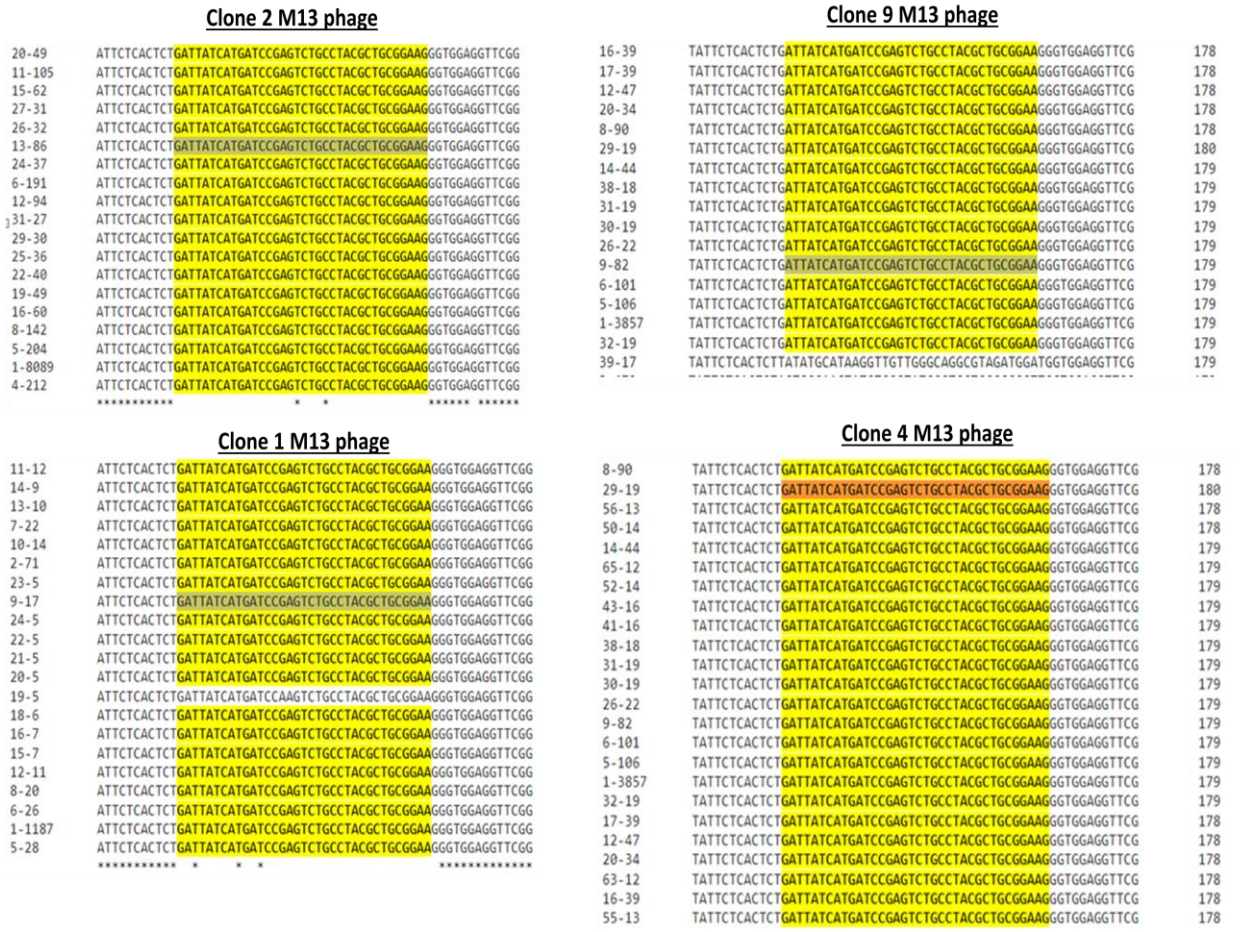


Figure 17: DNA alignment of the obtained NGS sequence data showing the most abundant DNA insert in M13 phage. It is worth to mention that in each analyzed clone this insert was repeated thousand times.

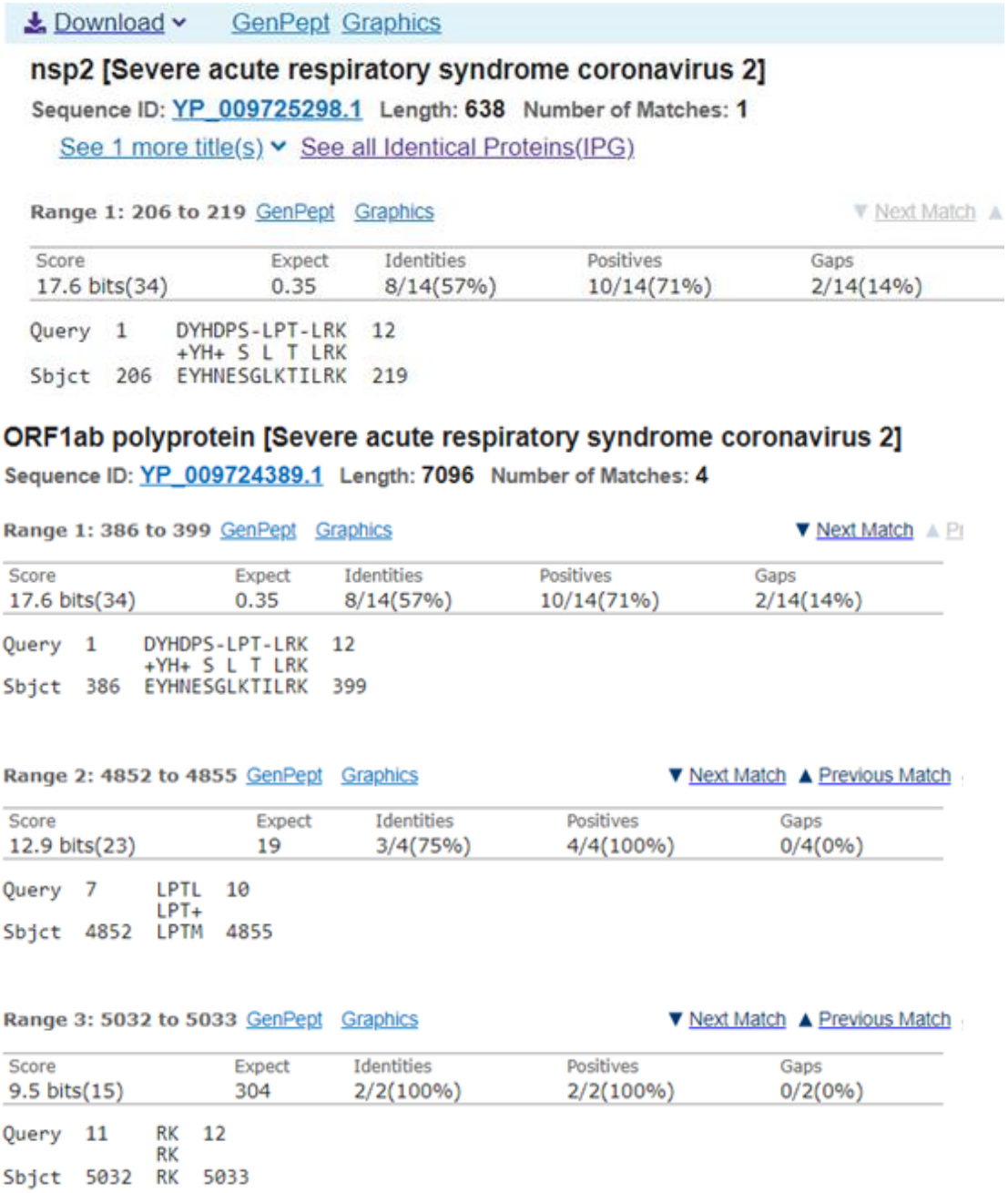


Figure 18: Protein BLAST analysis for the most dominant peptide that was identified by NGS sequence analysis.

Chapter four: DISCUSSION

COVID-19 infection can be detected, characterized by the monitoring the evaluation of Secretory antibodies produced against virus antigens, after binding of the virus with ACE2. IGG, IGM produced to neutralize, opsonize and prevent spared to non-Infected cells. A strong peak can of IgM on fifth day IGG on 10 days after disease onset(Fergie and Srivastava 2021). Specific IGG produced from B cell are detected on 7-10 days of infection, response against SARS-CoV-2 epitopes are the main reason to eradicate virus and prevent viral sepsis. A study on 36 convalescent patient confirmed that humoral immunity and cellular immunity CD4+ and CD8+ react against SARS-CoV-2 Nucleocapsid N protein efficiently(Le Bert, Tan et al. 2020).

Presence of an antigen inside a host cell evoke innate immune cells like Natural Killer and macrophage via chemo-attraction to recruit on infection cite, these cells take part of presented foreign body to present it on lymph-node. Lymph-node contain naïve B and naïve T cells, T helper2 cell attract to foreign body and produce activators for naïve B cell, this helps in increasing receptor-receptor binding between T helper2 and B cells. Important co-stimulatory signals include binding of TNF to TNF receptor, CD40 to CD40 ligand, and B7 to CD28(CHAPEL, HAENEY et al. 2013).

When B cell recognize foreign body, an iso-switching in Fab region of IGGs change binding affinity of IGGs to be complementary with epitope of antigen. Highly produced IGG define seroconversion on serum of patients infected with COVID-19. Based on Fab region, antibodies bind, neutralize and opsonize COVID-19 to activate complements which in result Eradicate virus(CHAPEL, HAENEY et al. 2013).

At the same manner T helper1 recognize presented foreign antigen on lymph node, cell activated then produce IL-2, IFN-γ and TNF-α which activate cell-mediated immunity via T cytotoxic CD8+ cell. Although Cellular immunity benefit of eradicate virus, but the destruction of host infected cells can cause serious damage for vital cells(CHAPEL, HAENEY et al. 2013).

After eradication, Antibodies, activated T cytotoxic, activated T helper and plasma cell are down regulated via IL-4 secreted by Th2 cells down-regulates the expression of IL-12 receptors on Th1 cells making them unresponsive to IL-12 inhibiting the production of IFN-γ and nitrogen oxide(Yao, Li et al. 2005)

There is no enough data about COVID-19 pandemic, almost all tests are designed based on primary data collected, and using of SARS-CoV-1 gene primers on SARS-CoV-2 PCR test.In this study a positive IGG against COVID-19 are collected as serum from Hebron for symptomatic patient with more than 2 weeks disease onset.

Binding of antibodies in patient serum with random expressed DNA segments on m13 bacteriophage that fused with p III seen on Western blot, as result humeral immunity secret IGG(based on study method) against COVID-19 in high number.

Phage display technology has been used for epitope mapping, this include selecting of linear epitopes, nonlinear epitopes and non-protein molecules also (Adda, 1999).

In Palestine a complete dependent on imported kits for identification of COVID-19 epitopes, phage display offer a billons of expressed variants on surface of bacteriophage, facilitate selecting and binding with target molecule. Panning is the process of selecting specific antibodies, this mean that antibodies are detected using their affinity with the correspond antigen, isolation of antibodies generally involved in repeated panning rounds. In each round bounded antibodies are enriched (phage too) and non-bound are washed away .So M13 are COVID-19 surface alternative and the antibody of patients are the correspond part that complete the reaction. Cloning sites have been introduced at the 5´ end of gene III for display of short peptide sequences as N-terminal pIII fusions, this meaning that gene III are the contact (receptor) point with target molecule (Antibody)(Smith and Petrenko 1997).

Phage display technology is a powerful method of epitope mapping that has been used to select peptides capable of mimicking linear epitopes, nonlinear epitopes, and even non-protein molecules. Different studies have been performed to determine whether mimotopes selected from phage display libraries by protective antibodies or antibodies against protective antigens in a variety of pathogens could serve as the basis for a peptide vaccine or a diagnostic tool.

Based on the fact that short peptides could mimic the binding sites on proteins, a phage expression vector that could display foreign peptide fragments as protein epitopes on the surface of phagotopes were developed. Several groups were then able to produce large diverse phage libraries, with random inserts of 12 amino acids. Such epitopes can be used for the mapping of antibody epitopes. Most phage display libraries consist of short peptides or protein fragments expressed on the surface of M13 filamentous phage as N-terminal

fusions to the minor capsid protein pIII or the major capsid protein pVIII. There are four or five copies of pIII protruding from one end of the phage particle, and up to several thousand copies of pVIII, the major phage coat protein. The advantage of pIII is that quite large peptide and protein inserts can be incorporated into it without loss of phage infectivity. Two pIII phage display libraries have been used in this study with the peptides expressed at the N-terminus of pIII coat protein of M13. The Ph.D-12 random peptide phage library (New England Biolabs, Beverly, MA) was used, this library contained 1.5×10^{13} pfu/ ml with a complexity of 2.7×10^{9} transformants.

One major application of random phage display peptide libraries is to determine the epitope of an antibody. Antibodies recognize small peptide motifs based on only three or four conserved residues. Based on the epitope motif identified by phage display, it is possible to delineate the region of a protein recognized by the antibody. An antibody can either be directed against a linear amino acid sequence in a protein or a discontinuous epitope, which has a distinct conformation formed by the protein folding. Phage library screening with such antibodies has yielded peptides that mimic the structure of a folded protein. These ligands are called mimotopes.

We select in this study 12 amino acid sequence library (a 12 amino acid expressed peptide) depend on B cell prediction bioinformatical study that shows a good numbers of COVID-19 epitope between 8-20 amino acid. On first reaction 50 active clone react against pooled serum are chosen, clones are purified then amplified one more time inside XL1-blue bacteria, through 3 panning, 8 clones are selected and amplified via PCR thermocycler, products run on 2% agarose gel electrophoresis and then NGS done for the 8 clones. Analyze DNA insert in sequenced clones shows in Phage2-s376 >10,000 repeat alone of an insert and >10,000 repeat for same sequence.

This result mean that this sequence are major content of the whole protein, so to ensure that, sequence then are translated to 12 amino acid query sequence, BLAST showed 57% identity in SARS-COV-2 epitopes, 8 identical amino acids are identical in NSP2, ORF1ab polyprotein and ORF1a.

DNA repeat in clones shows strong reaction for local patient serum with peptide variants expressed in it is conformational fold fused with p III on the surface of M13 bacteriophage,

positive clones reflect an epitope that may need more to be synthesized to become diagnostic rapid tool for investigation of positive IGG serum for SARS-COV-2 patient.

Conclusion: The first thing to think after this experiment is to how to make these results with benefit? Easily the highly reactive epitopes that shows a high reactivity in ELISA, DOT-ELISA and alignment represent the most of positive serum molecules which give us a good interpretation for the prevalence peptides among patients in Palestine. This may open a way to diagnose the presence of IGG antibodies in COVID-19 patient via conjugate the fused segment with albumin protein, we also can synthesis the query amino acid and conjugate it as vaccine with adjuvant.

ملخص باللغة العربية

العنوان: استكشاف الخارطة الجينية لسطح فايروس CoV2 SARS بإستخادم أمصال مرضى الفايروس والاستعانة بتقنية المكتبة الخاصة بفيروس الفاج M13

اعداد: تامر ابراهيم عيسى شبانه

اشراف: د. ابراهيم عباسي, د.رسمي ابو الحلو.

ملخص

مقدمة: ظهر فايروس كورونا المستجد في اواخر العالم 2019 حتى يومنا هذا,حيث يعتبر علامة فارقة في تاريخ البشرية الإنسانية, وقد سبب الفايروس العديد من الازمات في العالم وإغلاق كامل مناحي الحياة في كل العالم هذا وقد سبب العديد من الاصابات وحصد الأرواح, حيث اعتبرت منظمة الصحة العالمية هذا الفايروس على انه وباء ويجب على كل دولة في العالم تطبيق الخطط والبروتوكولات الخاصة بها لتفادي اكبر قدر ممكن من الاصابات.يسبب فايروس كورونا المستجد التهاب رئوي حاد سرعان ما يتطور ويسبب حالات تخثر الدم وجلطات تؤدي الى الوفاة وفي خضم ذلك تعتمد الاستجابة ضد الفايروس على مناعة المصاب ومدى تفاعل جهاز المناعة مع الفايروس,تشكل الامصال والاجسام المضادة مركز اهتمام في هذه الدراسة حيث تم اخذ امصال مرضى مصابين في فايروس كورونا من مستشفيات وزارة الصحة الفلسطينية وتم تحديد من خلال تقنية المكتبة الخاصة بفيروس الفاج M13 ماهية وترتيب البروتينات الخاصة بفايروس كورونا المستجد لدى المرضى.

طرق البحث: تقنية المكتبة الخاصة بفيروس الفاج M13 ساهمت وبشكل كبير في اكتشاف السطح الخاص بفايروس كورونا المستجد لدى المرضى وذلك بعد المرور بالعديد من الخطوات التأكيدية لوجود الاجسام المضادة في امصال المرضى حيث تم الاستعانة بتقنية الاليزا النقطية والفحوصات المصلية, وبعد ان تم التأكد من وجود الامصال, كان لا بد من وضع الامصال مع المكتبة الخاصة بفيروس الفاج M13 المتواجد داخل بكتيريا خاصة لمضاعفته, بعد ذلك ظهرت نتائج الارتباط مع الامصال وبينت ماهية وترتيب البروتينات على سطح فايروس كورونا المستجد.

النتائج: اظهرت النتائج الاولية تكرار احماض امينية في جميع الفاج M13 بكميات كبيرة,تم اخذ هذا الترتيب وترجمته الى بروتين بإستخدام ادوات علمية موثوقة حيث اظهر في نهاية ذلك ان هذا الترتيب من الاحماض الامينة يُظهر ويشير الى نوعين من البروتينات الموجودة على سطح الفايروس المستجد ما يثير الاهتمام ايضاً انه تم تكرار هذا الترتيب بكميات لا يمكن تجاهلها وقد تشكل النسبة الاكبر من البروتينات على سطح فايروس كورونا المتواجد لدى المرضى في فلسطين.

الاستنتاج: تُشكل الدراسة للبروتينات لدى مرضى مصابين في البلاد امراً مهماً لمعرفة ماهية وطبيعة الفايروس لمعرفة كيف يمكن للفايروس ان يهاجم جسم المرضى, تكرار الاحماض الامينية بتقنية فايروس الفاج M13 اظهرت نتائج مقبولة علمياً قد تتطور لتُعطي فحصاً خاصاً يكشف عن وجود الفايروس داخل البلاد.

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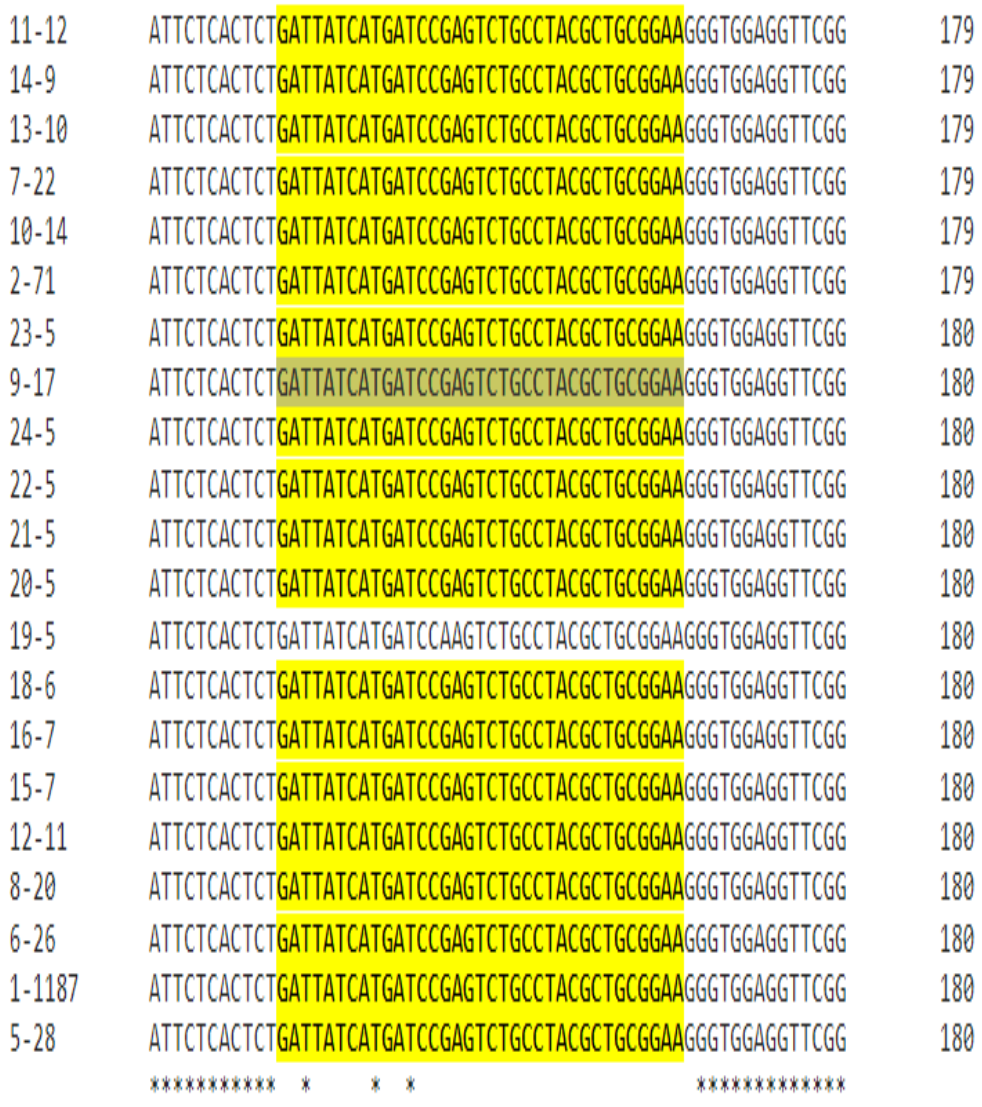
Appendix A
DNA alignment using clustal omega:

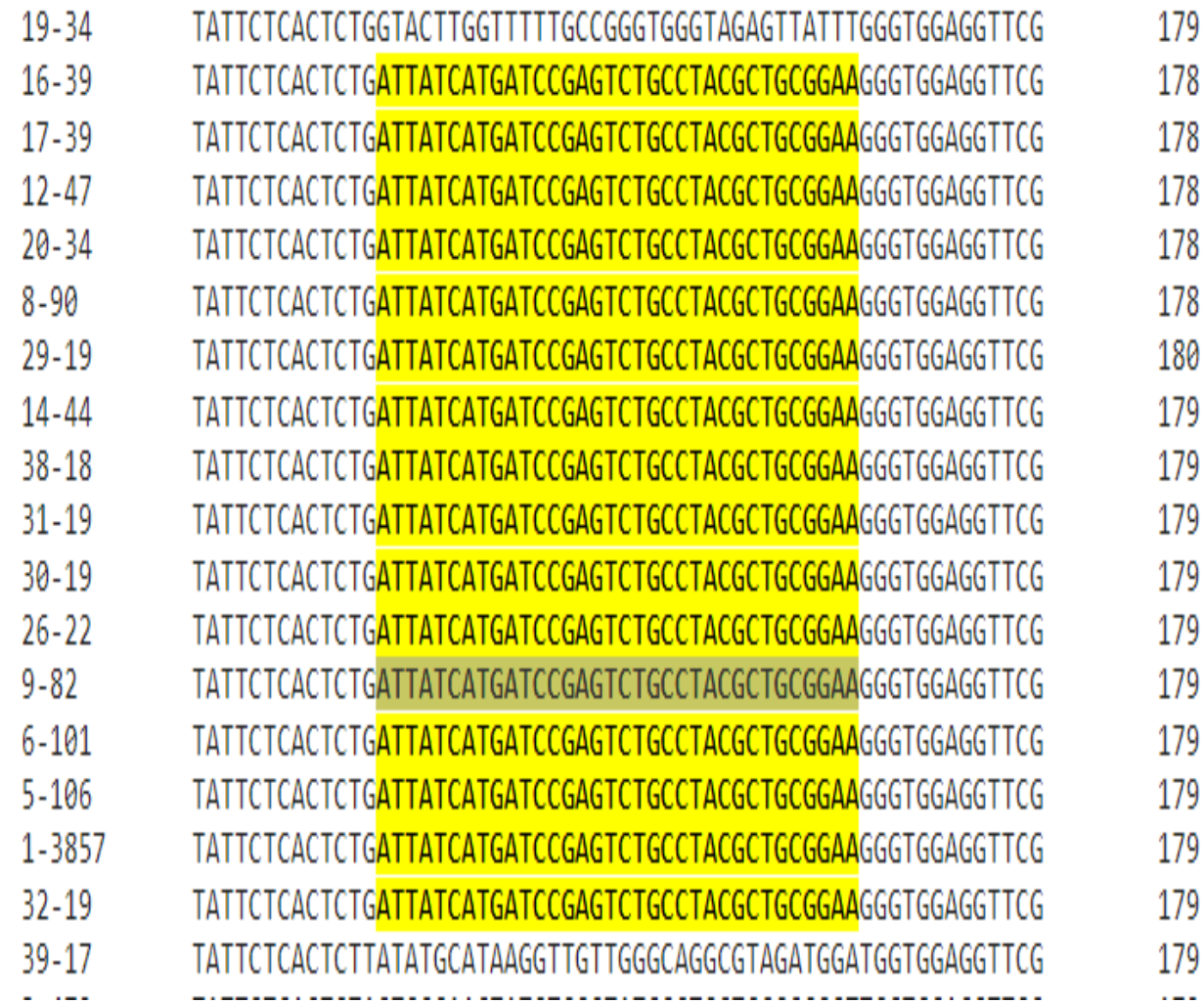
Phage2-s376 represent alignment result of repeated DNA insert.

```
23-37       ATTCTCACTCTCATGTTTCTACTACTGATTTGCTTGGGCCTCGTCGTGGTGGACGTTCGG       180
7-171       ATTCTCACTCTCAGGTGAATGGTTTGGGTGAGCGGAGTCAGCAGATGGGTGGAGGTTCGG       180
20-49       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       179
11-105      ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       179
15-62       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       179
27-31       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       179
26-32       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       179
13-86       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       179
24-37       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       179
6-191       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       179
12-94       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       180
31-27       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       180
29-30       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       180
25-36       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       180
22-40       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       180
19-49       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       180
16-60       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       180
8-142       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       180
5-204       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       180
1-8089      ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       180
4-212       ATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCGG       180
            ***********              *   *            ****** ******
```

Phage1-s361 represent BLAST of repeated DNA insert.



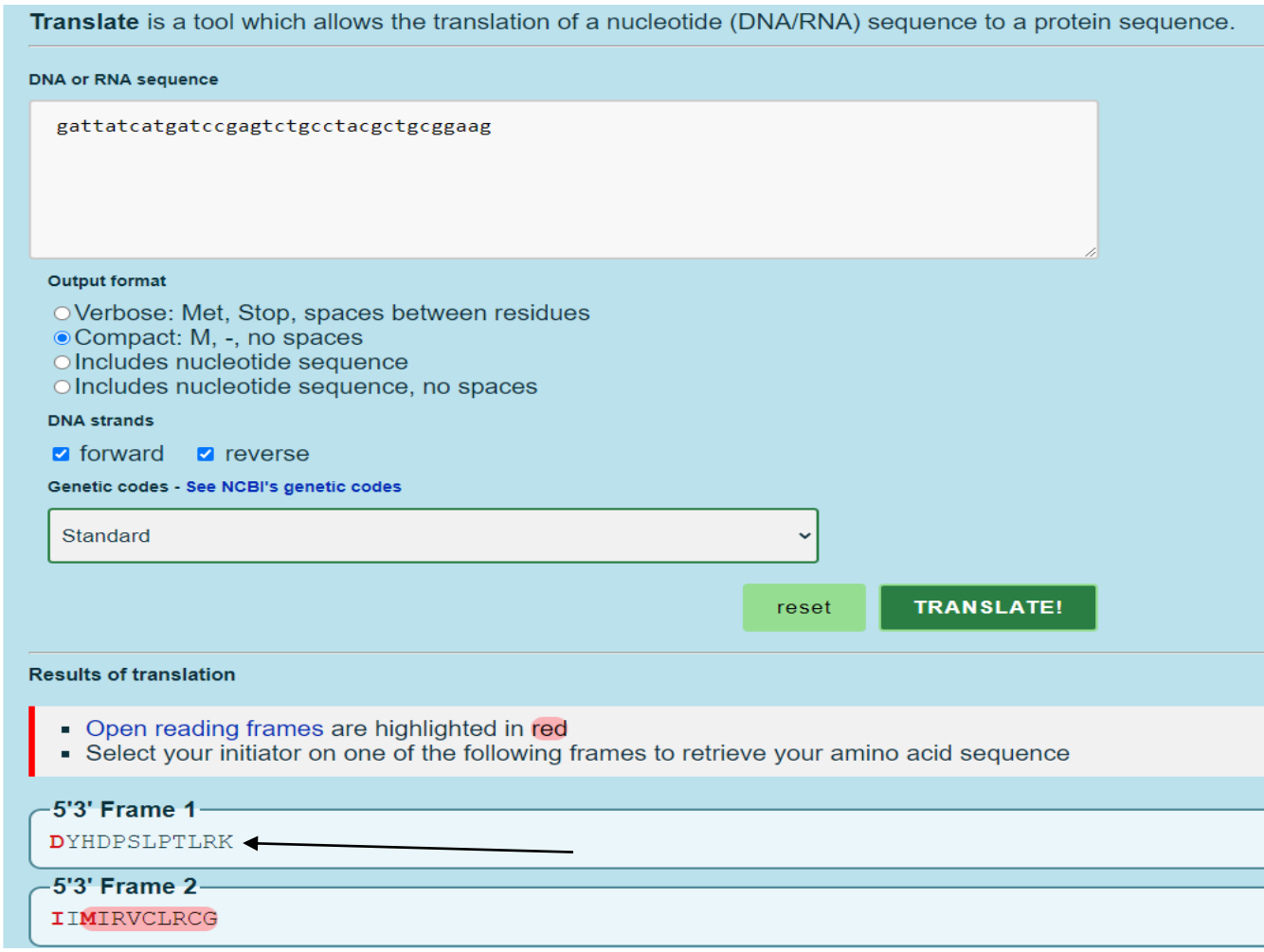
Phage 9-s378 represent BLAST of repeated DNA insert



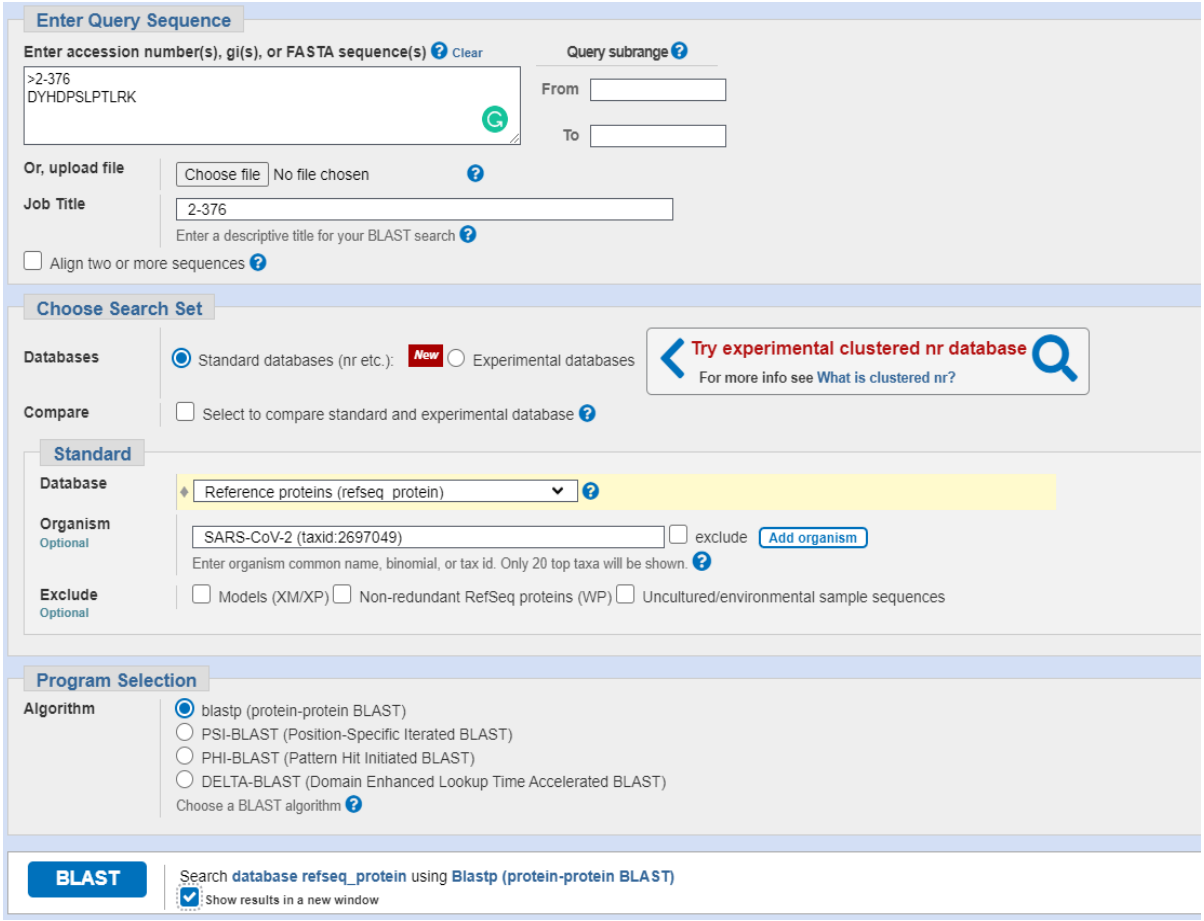
Phage4-s377 represent BLAST of repeated DNA insert

8-90	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	178
29-19	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	180
56-13	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	178
50-14	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	178
14-44	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
65-12	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
52-14	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
43-16	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
41-16	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
38-18	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
31-19	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
30-19	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
26-22	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
9-82	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
6-101	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
5-106	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
1-3857	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
32-19	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	179
17-39	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	178
12-47	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	178
20-34	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	178
63-12	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	178
16-39	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	178
55-13	TATTCTCACTCTGATTATCATGATCCGAGTCTGCCTACGCTGCGGAAGGGTGGAGGTTCG	178
68-12	TATTCTCACTCTTCGGGTCATGGGTCGAGTTTTAAGCCTGCGGTGCCTGGTGGAGGTTCG	179

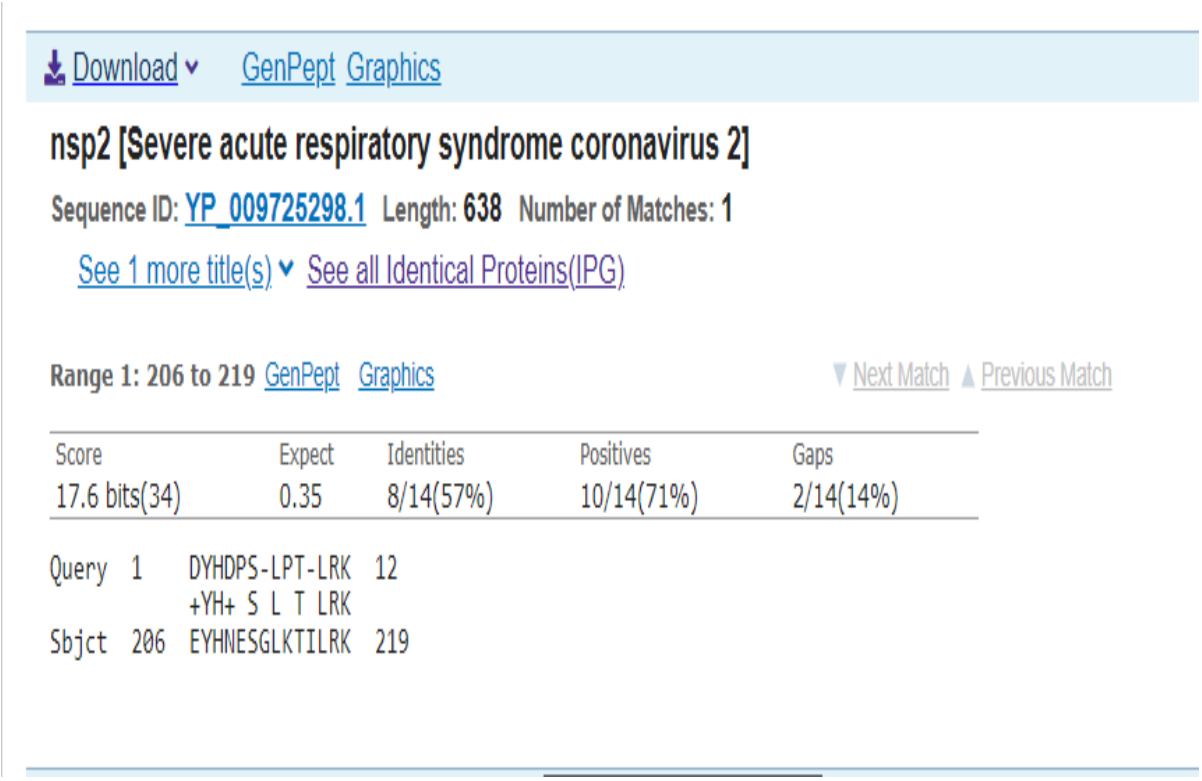
DNA insert translation using Expasy tool



Amino acids query BLAST



BLAST results of query amino acid sequence:



ORF1a polyprotein [Severe acute respiratory syndrome coronavirus 2]

Sequence ID: YP_009725295.1 Length: **4405** Number of Matches: **2**

Range 1: 386 to 399 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Positives	Gaps
17.6 bits(34)	0.35	8/14(57%)	10/14(71%)	2/14(14%)

```
Query  1    DYHDPS-LPT-LRK  12
            +YH+ S L T LRK
Sbjct  386  EYHWESGLKTILRK  399
```

Range 2: 4364 to 4365 GenPept Graphics ▼ Next Match ▲ Previous Match ▲ First Match

Score	Expect	Identities	Positives	Gaps
9.1 bits(14)	426	2/2(100%)	2/2(100%)	0/2(0%)

```
Query  9     TL  10
             TL
Sbjct  4364  TL  4365
```

ORF1ab polyprotein [Severe acute respiratory syndrome coronavirus 2]

Sequence ID: YP_009724389.1 **Length:** 7096 **Number of Matches:** 4

Range 1: 386 to 399 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Positives	Gaps
17.6 bits(34)	0.35	8/14(57%)	10/14(71%)	2/14(14%)

```
Query  1    DYHDPS-LPT-LRK  12
            +YH+ S L T LRK
Sbjct  386  EYHNESGLKTILRK  399
```

Range 2: 4852 to 4855 GenPept Graphics ▼ Next Match ▲ Previous Match ▲ First Match

Score	Expect	Identities	Positives	Gaps
12.9 bits(23)	19	3/4(75%)	4/4(100%)	0/4(0%)

```
Query  7     LPTL  10
             LPT+
Sbjct  4852  LPTM  4855
```

Range 3: 5032 to 5033 GenPept Graphics ▼ Next Match ▲ Previous Match ▲ First Match

Score	Expect	Identities	Positives	Gaps
9.5 bits(15)	304	2/2(100%)	2/2(100%)	0/2(0%)

```
Query  11    RK  12
             RK
Sbjct  5032  RK  5033
```

Range 4: 4364 to 4365 GenPept Graphics ▼ Next Match ▲ Previous Match ▲ First Match

Score	Expect	Identities	Positives	Gaps
9.1 bits(14)	426	2/2(100%)	2/2(100%)	0/2(0%)

```
Query  9     TL  10
             TL
Sbjct  4364  TL  4365
```