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Cloning and Expression of SARS COV-2 Surface Protein and its Use in Detecting Corona Viral Infections

RESEARCH

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A B S T R A C T

The main aim of this study was to develop indirect enzyme linked immune sorbent assay based on the use of bacterial cloned SARS CoV-2 spike protein as a cheap and continuously available source of antigen. This test was proved to be useful for a quantitative measurement and evaluation of antibody immune response among SARS-CoV-2 infected individuals. Standard cloning procedures had been used in cloning two different segments of the spike gene and its expression. The size of the two cloned segments were of 700 base pair and of 500 base pair, which were named clone 8 and clone 105, respectively. These DNA segments were cloned in pET28a plasmid and then expressed in Bl21 *E. coli*. Different serum samples were tested from: current, previous infection, vaccinated and non-vaccinated patients using the amplified expressed proteins in enzyme linked immune sorbent assay. The expressed proteins from each clone were responded with varying degrees of sensitivity against COVID-19 positive human sera, and we attempted to validate which of the two recombinant proteins is the best to be used in Corona IgG and IgM antibody detection. Based on the results of indirect enzyme linked immunoassay, most of the tested samples had greater antibody titers with clone 8, which was found to have a higher similarity (99% resemblance) to the severe acute respiratory syndrome coronavirus 2 surface protein using BLAST search. We recommend clone 8 with high potential to be used for large-scale screening for COVID-19 outbreak; nevertheless, it requires greater sensitivity and specificity validation.

Keywords: COVID-19, SARS CoV-2, ELISA, Cloning, Surface protein, Immunity

Introduction

COVID-19 disease devastated health and economy worldwide, challenged scientists for research and develop a fast and cheap diagnostic tests, in order to reduce the pandemic through proper diagnosis, medication and clinical quarantine (Chan et al., 2020). Serological tests are important for detection of the virus as well as detection of serum antibodies of infected people, especially for plasma donors, it could be used as qualitative and quantitative assay for the human immunity to COVID-19 (Amanat et al., 2020). Serological tests detect the existence of antigens as well as the detection of antibodies to SARS-CoV-2 as well as previous SARS-CoV-2 infection (IgG), and might help to confirm the presence of the current infection (IgM). For the detection of antibodies against SARS-CoV-2, several commercial and in-house serological tests based on recombinantly produced viral proteins nucleocapsid (N), surface (S), or epitope forms of the S protein have been developed. Despite the fact that different assays are applied, studies comparing multiple viral antigens in similar assays concluded that the S1 of the surface spike protein is an ideal antigen for SARS-CoV-2 diagnosis (Krähling et al.,

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2021). To develop a sensitive serological assay for COVID-19 there is a need to produce antigens in large quantity, thus using recombinant proteins expressed in prokaryotes like *E. coli* was vital in this experiment, which is characterized as quick and low-cost strategy. The developed serological test could be of great importance as a complementary diagnostic strategy to the well adapted Polymerase Chain Reaction (PCR) and mainly for follow-up of recovered patients, in addition to identification the asymptomatic individuals. In the present study, we reported the cloning and expression of COVID-19 spike protein segments, and the use of these segments in developing an indirect ELISA serological test for detecting SARS CoV-2 Spike protein IgG and IgM antibodies in serum samples.

Materials and Methods

Biological samples

Twenty nasopharyngeal samples of SARS CoV-2 confirmed infected individuals were collected from Bethlehem area. These samples were used to prepare cDNA material that was used for cloning purposes. Serum samples were collected from previously infected or vaccinated individuals. In general, pre-identified serum samples were obtained from Beit Jala Governmental Hospital, Beit Jala, Bethlehem. Ethical approval for the study methods were obtained from Al-Quds University Ethical Committee.

Cloning of SARS CoV-2 surface protein

The cDNA of positively identified samples were used as a source of SARS CoV-2 genetic material. The main cloning strategy was to amplify a segment of the surface protein using two designed primers and ligation of the amplified segment in a plasmid (pET-28a bacterial expression vector) according to the standard protocols that were provided by the plasmid manufacturing company (Novagen, USA). In short we designed two PCR systems in each two DNA primers (Table 1), that enabled amplification of two different regions in the COVID-19 surface genes (Tregoning et al., 2020).

Table 1: PCR systems used in cloning, the designed primers sequences and the used annealing temperature in PCR amplification

PCR System	Primer Name	Primer sequence	Annealing Temp
System 1	Cov19-1	Fwd-GAGCTCATGGCAGATTCCAACGGTACTATTAC Rev-GTTAATTTTCCTCTGGCTGTTATGGCC	53 °C
System 2	Cov19-2	Fwd-GAGTTTAATTTATAGTTGCC Rev-CTGTACAAGCAAAGCAATATTGTC	53 °C

PCR conditions

PCR reactions were carried out in 25µl reaction volume containing (2x ready mix *Taq* DNA polymerase mixture (Takara, Japan), and 10 pmoles of forward and reverse primers from each PCR system (Table 1). The amplified PCR product was analysed on 1.5% agarose gel electrophoresis.

Ligation and transformation of surface gene in pET-28:

Ligation of the amplified DNA surface protein segments was done in pET-28a expression vector at the restriction site XhoI available in the plasmid poly-linker cloning region. For this purpose, the amplified gene segments and the expression plasmid were cut by XhoI restriction enzyme (Thermo-Fisher Scientific, USA) and according to the manufacturer instructions. This was followed by ligation using T4 DNA ligase and according to standard kits and previously known standard methods (Thermo-Fisher Scientific, USA). The ligated products between the amplified DNA fragments and the pET-28a plasmid were transformed in *E. coli* bl21 using standard heat shock transformation method. The cloned gene segment was subsequently expressed as a fusion protein consisting hexa-histidine tag (His6-g gene-pET-28a) in *E. coli* BL21 cells. The transformed cells were grown on LB-agar plates containing 100 µg/mL kanamycin antibiotic. In order to check the presence of the cloned DNA segment in the plasmid vector and in the transformed bacterial cells, about 20 different bacterial cells were picked from the LB agar plate and transferred into 15ml tube containing 5ml LB media plus 50 µg/ml of Kanamycin antibiotics. The tubes were left to grow for overnight and the next day, 0.5ml of the bacterial cells were lysed by boiling and the clone was used in PCR reaction using T3/T7 universal primers in order to amplify the inserted segment if present in that specific tested recombinant clone.

Protein purification

The recombinant His-tagged surface recombinant protein of SARS-CoV-2 which expressed in *E. coli* was purified by two ways, one is by sonication, protein determination was carried out for the extracted proteins using spectrophotometer at 280nm. The other by Nickle beads affinity chromatography to purify His-tagged recombinant proteins. Histidine residues in the His tag

attached with great specificity and affinity to the empty sites in the coordination sphere of the immobilized nickel ions.

ELISA preparation:

ELISA assay started by plate coating with 100µl of 100 µg/ml nickel purified expressed surface protein diluted in phosphate buffer saline (PBS), plates were kept for overnight at 4°C. The tested serum samples were subjected to serial dilution in buffer (PBS, containing 5% foetal calf serum, and 0.1% Tween-20). The tested antibodies were incubated for two hours at room temperature followed by the addition of a secondary antibody (anti-human IgG or IgM conjugated with Horse-radish peroxidase (HRP)). The bound secondary antibody was detected by the addition of HRP enzyme ABTS substrate and later measuring the colour change using ELISA reader at 405 nm wavelength.

Results

Cloning of surface spike CoV-2 DNA segments: Initially five PCR systems were examined for the purpose of CoV-2 genes cloning, the primers of these PCR systems were covering about 500-800 bp segments from CoV-2 region located in a range of 25,000-27,000 of the genome, and that mainly code for the surface CoV-2 genes. Out of five different PCR systems; only the two above mentioned systems in Table 1 showed a successful gene amplification. These primers were able to amplify different positive CoV-2 cDNA material. The amplified bands by these two systems were grouped and used for the cloning in pET-28a plasmid. Cloning of PCR amplified DNA segments in pET-28a plasmid: As indicated in materials and methods, the successfully amplified DNA segments were ligated in pET-28a plasmid vector at XhoI site. After transformation in *E. coli* bl21 cells; different recombinant clones were obtained, which were indicated by successful growth of the transformed bacterial cells on LB agar plates containing Kanamycin antibiotic. Figure 1 shows the results of the amplified DNA segments that were successfully cloned in the plasmid vector, after their amplification using a universal primers (T3 and T7) located on both sides of the poly-linker cloning region in pET28a expression plasmid. In order to confirm the result and the presence of these inserts in the plasmid and without contamination by non-recombinant plasmid, some of these clones were streaked on LB agar plates with Kanamycin and Ampicillin and single colonies were obtained. The colonies were grown another time on LB media for overnight and retested for the presence of insert in these selected clones (Figure 2).

Figure 1: Agarose gel electrophoresis analysis showing the PCR products targeting different recombinant plasmids containing Cov-2 DNA insert segments. M: Size marker of 100bp ladder

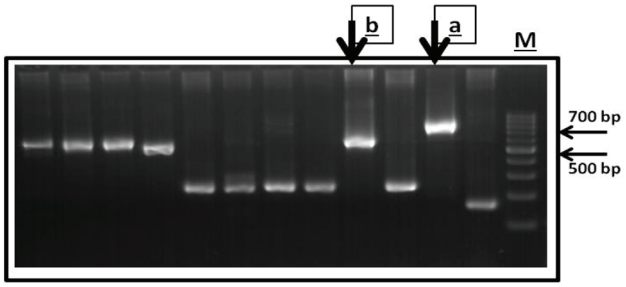

Figure 2: Agarose gel electrophoresis of some retested clones to check the purity of the recombinant plasmid in the bacterial cells. M: Size marker of 100bp ladder.

DNA sequence analysis of recombinant clones 8 and 105: The DNA segment in clone 8 and 105 were amplified and the PCR product was cleaned and subjected to DNA sequencing. The obtained DNA sequences were alignment with Cov-2 DNA segment (Wuhan-Hu-1), which part of the original Sars Cov-2 genome initially identified at the beginning of the current Cov-2 pandemic shows more than 95% homology between the sequenced clones 8 and 105 with reference DNA segment. Also, the obtained DNA sequence of both clones 8 and 105 were analysed by BLAST DNA analysis against different nucleotide entries in the GenBank. The analysis shows that both clones showed similarity with Cov-2 genome entries, the first identified hit namely was: (Severe acute respiratory syndrome coronavirus - 2 isolate SARS-CoV-2/human/USA/CA-LACPHL-AF08895/2021, complete genome / Sequence ID: ON422081.1).

Utilization of the expressed Cov-2 surface proteins in screening of corona related serum samples by Enzyme Linked Immunosorbent Assay (ELISA): Microtiter ELISA plates were coated with purified expressed and induced surface antigen protein. In each analysis; one plate was coated with surface antigen expressed by clone 8 and another plate coated with surface protein antigen expressed by clone 105. These ELISA plates were tested for their reactivity with serum samples that were used after applying serial

dilutions of the tested sera (1:50 1:100 1:200 1:400), negative control samples were used as well from freezing serum sample in Al-Quds University before the current pandemic. In each ELISA run, the cut-off value was determined by calculating the mean value of SARS Cov2-negative serum samples plus three standard deviations, readings above the cut-off value were considered as positive and were used to calculate the antibody titers Based on the obtained ELISA result readings, the results were used to illustrate the antibody titer results of each clone (8 and 105) as histogram, and this was in general according to the following criteria (current infection, previous infection, vaccinated and non-vaccinated). As can be seen from figure 3 and figure 4, that represents the average percentage reading of antibody titers for the different tested groups, it can note that the use of the expressed proteins from clone 8 showed more sensitivity than the use of expressed protein from clone 105 (Figure 3 and 4).

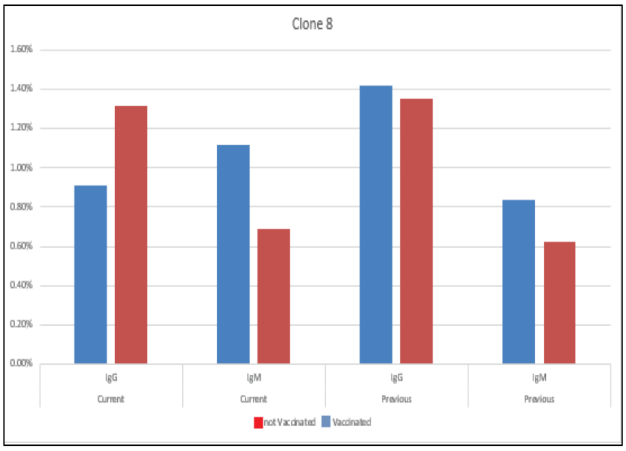

Figure 3: antibody titer results of clone 8 in general according to the following criteria (current infection, previous infection, vaccinated and non-vaccinated).

Also, it is possible to note that the produced antibody responses shown by this ELISA test for the previously infected individuals was much higher than the produced antibody responses seen among current infected individuals. This may relate to that: whether if patient may have vaccinated or not, or if the were previously infected and then they will produce more antibodies than those individuals whom were being recently infected and this is true especially with the IgG antibody (the main antibody class in secondary humoral immune response). Beside this, the data indicate that vaccination plays a major role to induce antibody production against the virus in both current and previously infected individuals, as it was noted that previous infection provides more immunity indicated by higher antibody titers of IgG antibodies compared to immunity with IgM antibody immune response (Figure 4).

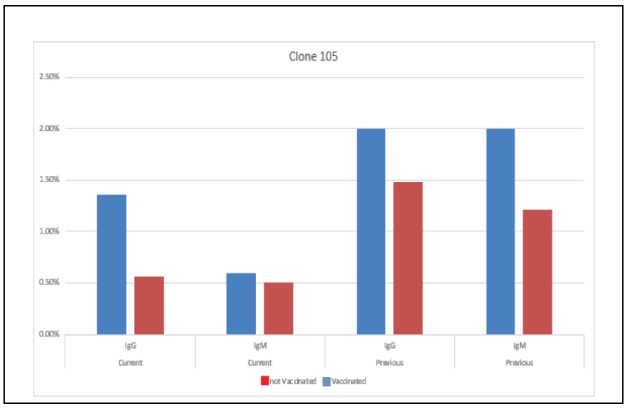

Figure 4: antibody titer results of clone 8 in general according to the following criteria (current infection, previous infection, vaccinated and non-vaccinated).

Discussion

The present study was performed to develop a suitable SARS CoV2 immunological test based on testing SARS CoV-2 IgG and IgM antibodies qualitatively and quantitively and to provide a continuous source of SARS CoV-2 surface protein antigenic material in sufficient quantities by using cloning technique to be used in our developed indirect ELISA tests. S protein is the most significant viral structural protein in terms of SARS CoV-2 pathogenesis because it promotes receptor binding and virus–cell membrane fusion and is rich in antigenic and neutralizing epitopes , so it was the most suitable antigen for such test (Dong et al., 2021). This study was also performed due to the public health concern about COVID-19 as the disease has been reported in almost all over the world. The results of the current antibody titers; utilizing the developed ELISA test; showed high IgG and IgM antibody titers among the Palestinian hospitalized population. The study also performed because of some imported commercial IgG/IgM test kits for COVID-19 had problems with sensitivity, resulting in high rates of false-negative test results, as the sensitivity and specificity of a fast antibody test for screening healthcare workers were studied in a prior study. In a certain study, serum of 389 health-care personnel who had been exposed to COVID-19 patients or who had symptoms was tested. All personnel were tested for SARS-CoV-2 on a monthly basis, with virus RNA detected by RT-PCR in nasopharyngeal swabs. After a median of 7.6 weeks, the Chemiluminescence Immunoassay (CLIA) and the Rapid test (KHB diagnostic kit for SARS CoV-2 IgM/IgG antibody) were used to identify IgG antibodies in serum. In COVID-19 positive persons, those with only

SARS-CoV-2 IgG antibodies, and those negative for both tests, the fast test was positive in 31/132 (23.5%), 16/135 (11.8%), and 0/122 instances, respectively. Specificity was 100 percent (CI95 percent 97-100) and sensitivity was 17.6 percent (CI95 percent 13.2-22.7) and 23.5 % (CI95 % 16.5-31.6). As a result, whether comparing Rapid test vs CLIA IgG or Rapid test vs SARS-CoV-2 positive RNA detection, Rapid test is not suitable for screening workers with past COVID-19 infection (CI95 % 97-100) (Filon et al., 2021). Our developed ELISA technique was used to determine the immunity by measuring the IgG and IgM antibody titers of the different tested serum samples against SARS CoV-2 crude antigen, these samples were collected from current positive infected patients (vaccinated and not vaccinated), negative infected patients (vaccinated and not vaccinated), and previous infected patients (vaccinated and not vaccinated), and the test had been reported to demonstrate high sensitivity and specificity when compared to other serological assays like western blot and chemiluminescence assay. The enzyme linked immunosorbent assay (ELISA) measures antibodies, antigens, proteins, and glycoproteins. Diagnosis of HIV infection, pregnancy testing, and detection of cytokines or soluble receptors in cell supernatant or serum are only a few examples. Because they rely on a pair of antibodies for capture and detection, immunometric ELISAs are highly selective. They are also thought to be compatible with a wide range of complicated materials, as they do not require sample extraction prior to analysis. The indirect ELISA method has a higher sensitivity than the direct ELISA method because it uses fewer labelled secondary antibodies to bind the main antibody. It is also less expensive than the direct ELISA approach since it uses less labelled antibodies (Butler, 2000). The immune system creates a wide range of antibodies in response to an illness, rather than simple one. Some of these antibodies attach strongly to an antigen, whereas others do not. Neutralizing and non-neutralizing antibodies are both types of antibodies. Antibodies known as neutralizing antibodies can "neutralize" viruses, as the name suggests. In reaction to SARS-CoV-2, certain neutralizing antibodies bind tightly to the coronavirus spike protein and prevent it from infecting the cell. Non-neutralizing antibodies don't accomplish this - or only do it in a restricted way - but they can nevertheless aid in the fight against viruses. Non-neutralizing antibodies do not protect infected cells against infection, but they can recognize viral antigens that are exposed or exhibited on infected cell's surfaces. Other elements of the immune system can come along and kill the infected cells when non-neutralizing antibodies bind to these surface antigens. COVID-19 neutralizing antibodies are tested in most labs because they are a good indicator of infection prevention. However, we don't know how high neutralizing antibody levels needed be to protect COVID-19 patients from infection or severe disease (Chvatal-Medina et al., 2021). Simple monitoring and assessing vaccine effectiveness studies has shown that as neutralizing antibodies decline, the chance of a breakthrough infection rises. Meanwhile, scientists rely on other signs to establish the efficacy of vaccines. Examining vaccination effectiveness in the real world, both in specific populations and across time, is part of this. When it comes to deploying COVID-19 boosters in the summer of 2021, Israel chose this technique. According to data from the country, infections detection were more likely among people who had been vaccinated earlier in the year than those who had been vaccinated more recently. You can't take an antibody test to see how well you're protected against the coronavirus after vaccination or a spontaneous infection because there is no connection of protection for COVID-19, taking into account the importance of cellular immune response in viral infections. (Krammer, 2021). ELISA test is considered a simple diagnostic immunological test that can be performed in many laboratories; not only in hospital and main health care laboratories but even it is used in many private laboratories. The only limitation of this specific ELISA tests is the production of pure recombinant antigen using nickel purification. Large amount of bacterial cultures is needed for this purpose and special technical efforts are needed from well trained individuals to perform such purification protocols.

Conclusion

A recombinant SARS-CoV-2 surface proteins were cloned and expressed and then used in the current investigation to show the design and development of a low-cost in-house ELISA. The assay could offer information about COVID-19 seroprevalence, which could be useful for disease prevention and control at the population level. Serological tests are essential for establishing SARS-CoV-2 exposure and protective factors. From the two clones that were constructed in the current study, it was noted that, the used expressed protein based on Clone 8 is likely more sensitive than the expressed protein based on clone 105. We recommend that this clone be utilized for large-scale screening of covid19 in any future outbreak in our nation; nevertheless, it requires more sensitivity and specificity validation. Despite we used a positive pool serum but we recommend to use positive known antibody titer from any other laboratory as positive

control next time, and we recommend to use both clones as combination in next study for validation or to use surface protein with other types of protein like nucleocapsid as it is more conserved gene with less mutations. We recommend as well to test specificity of our developed test by using other types of Corona viruses to increase validation.

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Author Contribution

Rasmi Abu-Helu: supervising the study and manuscript writing, Hana Al-Khatib conducting the experimental research and writing the manuscript, Tamer Shabana, Aseel Eqneiby, Rawan Ajlouni, all helped in sample collection and part of the conducting part of the experimental work. Ibrahim Abbasi: supervising the study and manuscript writing.

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