P(d)PANA: A phagemid vaccine design against COVID19

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ABSTRACT

The COVID19 pandemic in 2021 has strained the technical, scientific and industrial resources of every country around the world. However, few nations have been able to confront this pathogen under an adequate production, storage and distribution scheme for vaccines within and outside their borders. This has implied that certain territories a left behind in vaccination due to the lack of productive means and infrastructures necessary to address the previous points. One alternative that we offer with our research corresponds to the design of a universal, efficient and safe vaccine against COVID19 for emerging economies. All this under a bioinformatics approach for the selection of the antigenic targets with the best potential and their respective incorporation into the capsid surface of bacteriophage M13. Given the orientation of our work, this consists of a phagemid display as a peptide array for the development of neutralizing antibodies, abbreviated by its acronym as P(d)PANA. The basis of our design is to propose an alternative vaccine against coronavirus that is capable of providing not only immunity, but also a cost-effective production scheme, durable in storage and easy distribution to developing countries.

Key Words: COVID19, Vaccine, Phagemid, Phage.

INTRODUCTION

The COVID-19 pandemic affected everyone; the spread of the virus has caused a steep increase in fatalities worldwide as well as hardships in all aspects of society. The design of vaccines against SARS-CoV-2 is one of the main goals of global scientific research. From numerous studies on this disease, different approaches to immunization strategy for humans have emerged. Each takes advantage of the knowledge of molecular and cellular biology in their respective approaches, thus offering efficiency and safety in the respective administration to the public. However, all WHO-approved vaccines have faced a bottleneck due to technical limitations in their production, storage and distribution, from the simplest vaccines using inactivated coronavirus (Sinopharm, Sinovac) to the most complex vaccines such as those based on mRNA (BioNTech and Moderna) or viral vectors (Johnson & Johnson and Astrazeneca). Because of this, there is a priority to develop other processes for the manufacture, testing and distribution of new vaccines to ensure a constant supply of new doses for the population.

In developing countries especially, such conditions have been the bottleneck for vaccine accessibility. In Latin America, for example, access to vaccines is subject to additional problems caused by a lack of adequate infrastructure for storage and distribution of doses within the territories of each state. Therefore, alternatives capable of meeting the local needs of this region must proposed.

Our proposal addresses our national reality by considering all these elements, evaluating the vaccine design strategy, and adjusting to the prevailing conditions. Therefore, our research's objective consists of the design of a phagemid display as a peptide array for the development of neutralizing antibodies (P(d)PANA). This also considers efficiency parameters for the immune system response as well as biosafety parameters for the public.

One point to highlight is the Phage Display tool, which, although known within the field of molecular biology, its application as a means of vaccination against COVID-19 is just being evaluated, but with encouraging results (Staquicini *et al,* 2021; Kim *et al,* 2021).

Reasons for choose a peptide presentation system based on the M13 phage are due to the very advantages that this system offers over the other strategies mentioned above:

- Easy gene editing, the insertion of exogenous genes can be performed via PCR.
- Rapid production: Since it is a non-lytic phage, and its host is an E.coli, it facilitates rapid and inexpensive production (de Vries et al, 2021; González-Mora et al, 2020).
- High thermal stability: It does not require a cooling chain for storage, withstanding up to 70°C (Gonzáles-cansino et al, 2019)
- It does not require the use of adjuvants: The phage capsid proteins are capable of triggering an innate immune response (de Vries et al, 2021; González-Mora et al, 2020)

These advantages over other vaccine designs are what make phage-based vaccines the most cost-effective option for production, storage, and distribution. Motivated by this, our project focuses on optimizing such a system to be functional for the fight against SARS-CoV-

2, and that in terms of manufacturing it results in the most efficient to be easily reproducible on an industrial scale and at a lower cost.

P(d)PANA as a molecular biology technique is based on a informatics, immunologic, biochemistry & genomics approaches in which regions of the coronavirus spike protein with highest antigenic potential are selected and displayed on M13 phage envelope as a means of vaccination. Each details will be explained bellow

METODOLOGY & RESULTS

1. Massive data filtering

Given the need to generate a consensus spike protein sequence of the different SARCoV2 variants, GISAID.org used as a key database. It should note that the file downloaded from that website pertains only to sequences registered up to 28-07-2021. However, it should be noted that a large number of the sequences uploaded there present problems with the final availability of their data. In order to visualize this situation, free software Jalview has used as a reference, with which the following problems could detected:

- Duplicated sequences in amino acids
- Sequences with gaps between amino acids
- Incomplete sequences
- Sequences with errors and/or amino acid deletions
- Variable number of amino acids (excluding animal coronaviruses).

In view of this situation, a script elaborated considering the parameters of:

- Year of registration: for the purposes of the project, only sequences from the present year 2021 up to the above-mentioned date taken.
- Protein length: Only for 1273 amino acids.
- Number of continuous gaps: These represented by null characters "X", and only those with less than 10 continuous gaps are accepted.
- Percentage of gaps: Only those sequences with a value of up to 10% of "X" taken into account.

From such initial conditions, and from the subsequent application of the script on the downloaded data set, a high performance obtained for the visualization of functional sequences for the purposes of the project itself. From a 3.6 Gb fasta file with 2.6 million sequences it was possible to extract 57629 sequence in an 80.1 Mb fasta file. Latter file did read better by the Jalview software, and thus worked with it for the generation of the consensus sequence.

2. Immunoinformatic analysis:

The consensus sequence generated with the Jalview program used in the immunoinformatic servers for the antigenic analysis. The sections used to divide the sequence represent the glycosylation protein sites mentioned by Watanabe et al. (2020). The reason for grouping immunoinformatic analyzes in these categories is due to the masking effect that the glycan shield has on the surface of the spike protein (Casalino et al., 2020).

Key to the filtering and statistic representation of the data lies in the structure of the spike protein itself. Structure covered by sugars on its surface, also known as the glycan shield, which prevent adequate recognition by the immune system during the initial stages of infection. Therefore, the entire sialic protein is not functional for immunization; if the body were to establish a peptide that falls precisely on the amino acids covered within the glycan shield, it would be a deficient immunity. Thus, in order to identify the most useful peptides for the immunorecognition, the regions taken are those between glycosylation, since this favor the development of neutralizing antibodies in each individual. Likewise, given the objective of addressing the development of neutralizing antibodies, it has taken into account that the properties and peptides identified in the servers were from amino acids 1 to 583. This is because the S1 section of the protein is located in this region spike and is involved in the process of entry of the viral particle into human cells.

Immunoinformatic analysis shows 182 521 ligands for the MHC-I system, while 96 748 ligands for the MHC-II, both systems associated with the presentation of antigens to T lymphocytes. I addition, most of these peptides are located in the last group between glycosylations of the sequence, ranging from 343 to 583 aa´s. On the other hand, the results obtained for antibodies evaluated different properties of the sequence, being the last group with the highest weight and abundance of available epitopes. Then the risk of allergenicity of the identified peptides evaluated. To do this, the Excel table files converted to Fasta- type files by means of a Python script, each with 100 peptides per file. With this new data set, they manually entered into the Vaxigen server. In this step, the antigenic potential of the peptide sequences ruled out or confirmed. Accordingly, a new library generated with the non-allergenic sections of the consensus sequence.

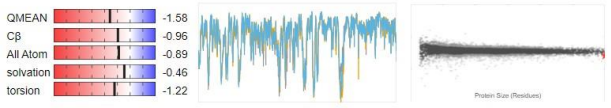
3. Consensus protein modeling

The objective of this stage was to demonstrate the structural stability of the consensus protein. For consensus sequence, structural modeling of the same carried out, using two strategies: By homology and De Novo. The first part of the modeling by comparison of sequences with structures already known in the Swiss-model servers. While for the second strategy used the AlphaFold2 server in Google Collab. From both methods, only one result obtained by homology, since, for the second tool, the resources assigned by the server were not sufficient for processing this protein. However, for the

project standards, the results offered by Swiss-Model were sufficient.

On the other hand, the sequence and structure of the consensus protein also compared with some of the spike proteins of the variants. From this result, it observed that there are no major changes in the consensus sequence or in its derived structure. This is because the same number of proline residues conserved with respect to the other variants. Therefore, it can be say that the 3D model obtained presents the necessary stable conformation

Model #01	File	Built with	Oligo-State	Ligands	GMQE	QMEANDisCo Global
	PDB	ProMod3 3.2.0	homo-trimer (matching prediction)	6 x NAG: 2-acetamido-2-deoxy-beta-D-glucopyranose; 9 x NAG-NAG: 2-acetamido-2-deoxy-beta-D-glucopyranose-(1-4)-2-acetamido-2-deoxy-beta-D-glucopyranose; 2 x NAG-NAG-FUC: 2-acetamido-2-deoxy-beta-D-glucopyranose-(1-4)-[alpha-L-fucopyranose-(1-6)]2-acetamido-2-deoxy-beta-D-glucopyranose;	0.73	0.74 ± 0.05



Template	Seq Identity	Oligo-state	QSQE	Found by	Method	Resolution	Seq Similarity	Range	Coverage	Description
7krs.1.A	100.00	homo-trimer	0.85	HHblits	EM	3.20Å	0.62	14 - 1162	1.00	Spike glycoprotein

Figure 1: protein swiss-model report for consensus protein

4. Standarization

Regarding the construction of the phagemid, our work based on the genome of the bacteriophage M13. It characterized by being a non-lytic type filamentous virus whose only host is E.coli (Rakonjac et al, 2011). This phage is made up of eleven genes arranged in a semi-continuous manner with 2 non-coding intergenic regions (IR) with promoter and terminator sources respectively, and 5 of these genes (pIII, pVI, pVII, pVIII, pIX) give rise to capsid proteins (van Wezenbeek et al, 1980).

Our phage modifications corresponds to the insertion of two new genes and the change of an existing one. The incorporation of these new DNA sections proposed to be by the exponential cloning method by megapriming PCR (Ulrich et al, 2012). The reason for choosing this method over others is due to the limited insertion space, since it falls within a limited range within the IRs of the genome (Horton et al, 2013; Mathieu et al, 2014).

From M13 phage genome sequence proposed by van Wezenbeek et al (1980), each gene sequence tagged, including two of interest for the purposes of the project. These correspond to genes VII and IX, both whose proteins are part of two of the five phage capsid proteins, and which have a potential for peptide presentation (González -Mora et al, 2020).

Additionally, it desired to preserve phage infectivity of the once purified. Reason why capsid protein III was not taken into account, despite being a popular system to express proteins of interest (POI) in its sequence, but whose main function is to anchor and enter E.coli (González -Mora et al, 2020).

However, the limits of insertion of peptides in the key proteins mentioned above must considered. The first barrier lies in the insertion capacity of new amino acids in pVIII and pIX, with up to 10 amino acids and 1000 amino acids respectively. However, it also influences the amount of proteins available in the virus capsid, where pVIII has up to 2700 copies and its counterpart pIX only has five copies. Given this duality of aa's length and number of copies, the antigens that are exhibited on its surface should be prioritized (González-Mora et al, 2020). On the other hand, another influencing barrier is the genomic stability that these new recombinant genes and the sites where they inserted may have. Since, during the process of replication of the phage in its host, losses of the new genes can caused (Enshell-Seijffers et al, 2001; Horton et al, 2013; Mathieu et al, 2014).

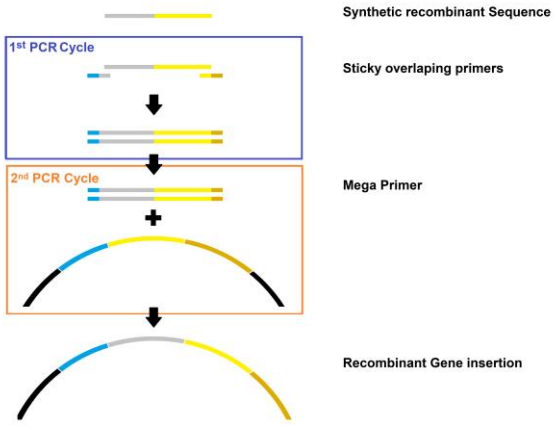


Figure 2: Megapriming protocol shechem

According to the immunoinformatic and structural analyzes of the consensus sequence, antigens presence correspond to those identified within the region 343 to 583 from it sequence, and that correspond to the receptor binding domain (RBD). Based on this, it was decided that for the pVIII protein a non-allergenic and 12 aa antigenic peptide from the aforementioned region would be used, while for pIX the entire domain itself would be used, since it has a globular-like structure in its structure.

An important point to highlight is that the insertion of new sequences within those that encode pVIII and pIX obey a series of parameters for their correct incorporation, and of which following will mention:

a) pVIII:

- **Modification at the C-terminal:** The insertion of any motif other than the base sequence be made at the carboxyl terminal end of the polypeptide, due to the way of assembly in the virus capsid (Enshell-Seijffers et al, 2001)
- **Two classes of pVIII:** Being the protein with the highest abundance in the capsid, its modification can affect the stable form in which they are incorporated into the virus capsid, which is why the support of the helperphage with a wild type copy is needed, or the same phage with a wild type sequence and a recombinant one (Wang & Yu2004).
- **A synonymous sequence:** In the case of using the strategy of a phage with two pVIII genes, the recombinant region must have a synonymous sequence for the base of pVIII. This is because spontaneous deletions can be generated during the replication of the virus if it has two exact copies of the same gene (Enshell-Seijffers et al, 2001)

a) pIX:

- **Modification at the N-terminal:** make insertion of any motif of the base sequence at the initial aminoacyl end of the polypeptide, since it corresponds to the surface exposed towards exterior of the capsid (Mohammadi et al, 2016; Tornetta et al. al, 2010). Likewise, the insertion must be after the Methionine and Serine codons respectively (Nilssen, 2011)
- **Flexible linker:** it corresponds to a peptide sequence of no more than 20 amino acids that acts as a bridge between POI and pIX (Mohammadi et al, 2016 ; Nilssen, 2011).

Finally, the last gene of interest is the tetracycline resistance gene. A sequence already standardized within the iGEM community and that has its own promoter and terminator domains (Balley, 2018). This genetic piece corresponds to the metabolic marker for the conservation of the M13 phage genome within *E.coli*.

5. Genome design

Based on all of the above, a phagemid with the two recombinant proteins (pVIII and pIX) and the tetracycline resistance gene (TetR) chosen as strategy. In the case of pVIII, it decided to leave Wild type DNA sequence and add a synonymous sequence of nucleotides + antigenic peptide nucleotides. Now, for pIX, its approach was different, because it involves only the modification of the N-terminal end of its amino acid sequence, therefore, only the linker sequence from Mohammadi et al (2016) + RBD inserted.

For the construction of the pVIII codon synonymous sequence, the original sequence provided by van Wezenbeek et al (1980) and the GenScript (SF) codon frequency table taken as reference. Subsequently, the nucleotides organized by amino acids of pVIII with the synonymous codons for them (Table 3). Based on this, the codons were alternated, for the aa's with a single codon we proceeded to alternate with the next one, while for the aa's with 3 or more codons we proceeded to randomly select any of the options available for the construction of the synonymous sequence

To verify the similarity between the original and the new sequence, it was compared using the Pairwise Sequence Alignment tool from EMBOSS Needle (2021). The result provided by the server presented a 65.18% similarity regarding the alignment of the codons

In another vein, the peptide sequence for pVIII and the linker + polypeptide for pIX converted to *E.coli* nucleotides using the JCat tool (Groote et al, 2005). After obtaining the nucleotides for both antigens, they incorporated into the corresponding sections within the genes. In the pVIII gene it was incorporated before the TGA (Stop) codon, and for pIX it was inserted after the Methionine and Serine codons respectively.

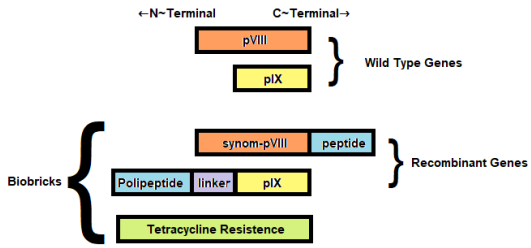


Figure 3: Schematic representation for key proteins

van Wezenbeek et al (1980) have given confusing numbering for the M13 phage genome, thus a new numbering system was developed according to the sequence provided by the same author. From this new order, each of the genes and regions of the virus identified according to the sequences of the genes identified by Suggs & Ray (1979), van Wezenbeek et al (1980), Kim et al (1981), Russel & Model (1989), Schaller, H. (1979) and Smeal et al (2017). All work made with Benchling digital tool, from which the following figure obtained:

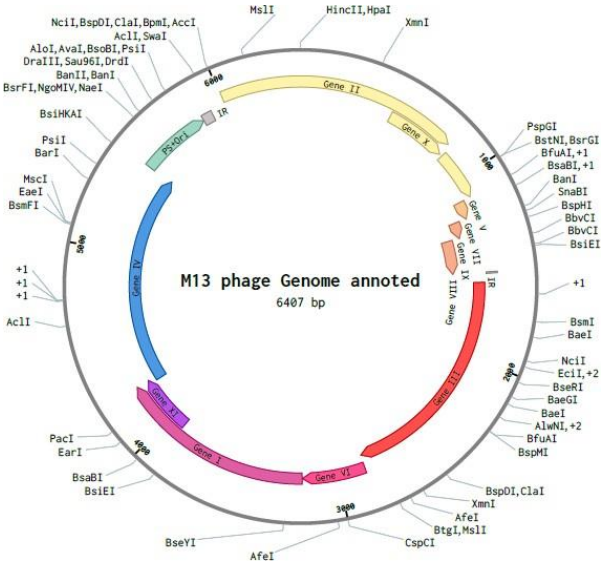


Figure 4: M13 Phage genome

Once each of sequences pVIII, pIX and TetR were define, we proceeded to design their constructs to make the Bioparts. As mentioned in the previous point, the insertion of foreign genes into the M13 phage genome will carried out by means of the TAL technique. According to this protocol, it requires the incorporation of flanking primers of the exogenous gene, and that they recognize specific domains of the genome for incorporation (Horton etal, 2013; Mathieu et al, 2014).

According to Mathieu et al(2014) recommendations, the design of these primers follows those of a conventional PCR, but with the difference that they must contain an affinity section to the gene of interest and another alignment section for genome recognition. Therefore, for each of the aforementioned genes of interest, we proceeded to design the respective primers with dual functionality. All this was done by selecting 5'→3' regions of the aforementioned intergenic regions, for subsequent incorporation as ends of each of the external genes.

Table 1: Primers for ovelaping insertion for each gene

Sequence Name	Primers (5´→3´)	Self-complementarity	Self 3' complementarity
pIX-R	**Forward:** CGCTGGGGTCAAAGATGAGT	3.00	1.00
	Reverse: TGAGGAAGTTTCCATTAAACGGG	6.00	3.00
pVIII (Wild Type)	**Forward:** TGGCATTACGTATTTTACCCGT	6.00	2.00
	Reverse: CGGATGCCTTTAACACTAGACT	4.00	3.00
SpVIII-R	**Forward:** GTGTTAAAGGCATCCGTGGC	4.00	2.00
	Reverse: GAGCCTTTAATTGTATCGGTCAG	4.00	1.00
TetR	**Forward:** TCTGATTTAAACCGGTCTCGGG	6.00	2.00
	Reverse: TCGCGTTAAATTTTTGCTCAAAGC	6.00	3.00

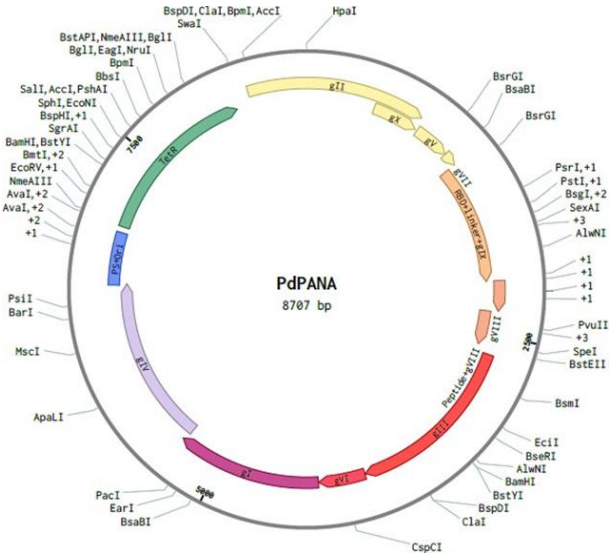


Figure 5: Phagemid

6. Replication of the genetic material of Sars-CoV-2 on the strain DH5*a*F' of Escherichia coli

To insert the genetic material of Sars-CoV-2 inside Escherichia coli bacteria, the virus needs to become a bacteriophage. To achieve this, it is necessary to realize a recombinant homology process between Sars-CoV-2 and the bacteriophage M13. The recombinant homology allows the exchange of the genotypic and phenotypic characteristics from both virulent structures to obtain the organism expected (Chen et al., 2019). Which would be the phagemid based on bacteriophage M13 modified, so it has spike protein in its capsid (affecting in the least the proteins that allow any metabolic process). Thus, the genetic material found on the capsid's interior would be a hybrid between both virulent organisms. Moreover, the molecular markers that are being transferred are: the resistance genes to the tetracycline (tet) and the modified genes that correspond to the phage, protein VIII and IX.

Nevertheless, in proof no producing a bacteriophage that could attack the strains of E. coli found in the digestive microbiota, which could have a negative effect on the activation of the T regulatory cells (Maldonado, 2021). E experimentation will be with the strain DH5*a*F' because is a modified version of DH5*a* (Kostylev et al., 2015), one of the most common strains of this bacteria that is utilized in laboratory work. So, it does not represent any health security problem at first sight. The difference between both strains is that DH5*a*F' have a pili and its antecessor does not. The selection of a strain with pili is important because it allows an easier interaction between the bacteriophage and the bacteria (Chen et al., 2019).

7. Design of the bioreactor

To design a reactor is necessary to understand the productive process that will be made and how the parameters that affect the process development will be measured (Rojas y González, 2011). Knowing this, the fermenter will be a stirred tank of 14 liters. At the bottom of the tank will be a magnetic surface that with a magnet will mix the tanks' contain. Additionally, to guarantee the use of the correct operation conditions (temperature, pression, pH, and others) there will be a constant revision in the tank with the use of measurement instruments for each variable. There will be a thermometer, a pH meter, a barometer, and a probe of O2 and CO2. There will only be one measurement instrument for each variable because the mixing in the tank will guarantee a unique measure of every parameter at the time of revision.

- When the sensors perceive an out-of-range lecture will give the following answers:
- When the temperature is not the expected one, a heat exchange blanket will be turned on. If the temperature is below the range, the heat exchange blanket will have hot water in its interior, in contrast, it will have cold water when the interior's temperature tank is higher.
- When the pH is not the expected one, a buffer solution will be released at the necessary volume to establish the correct pH. If the pH is lower, a based buffer solution will be released. If the pH is higher, an acid buffer solution will be released in the interior of the reactor.

The pressure tends to increase in a reactor because the organisms are producing some gases like O2 or CO2 (the production depends on the organism utilized). When the sensors perceive a high concentration of products that could harm the bioprocess or a higher pressure than the expected one a valve will open, and it will release the production gases.

Moreover, the bioreactor will have an extraction system that goes from the fermenter to the filtrate system to allow the extraction of phages in a secure way.

8. Fermentation conditions

The experimentation will be in anaerobic conditions to avoid lysis on the cell walls of Escherichia coli, which provide a better and correct interaction between the cell walls of the bacteria and the bacteriophage. Also, this bacterium needs a culture medium that has a carbon rich fount and an antibiotic to avoid the growth of an unwanted microorganism (Hernández, 1974). The process will be at atmospheric pressure, under a pH that varies between 6 and 7.5, and at two different temperatures in the different phases of the productive process.

The culture will have two phases. The first phase is when the bacterium is being inoculated in the culture medium, the inoculated concentration needs to be defined in an experimental way. The culture medium is conformed with

glucose, tryptophan and tetracycline. According to the bibliography, 15 µl of Escherichia coli DH5*a*F' submerged in a broth rich in tetracycline should form colonies after an incubation of 16 hours under a 37ºC temperature (Biolabs inc, 2016).

After an optimum bacteria production is reached, the temperature will drop to 29ºC because at this condition exists a metabolic balance between the bacterial and viral growth (Warner et al. 2014). Next, the bacteriophage M13 modified will add to the fermentation and it will inoculate for a determinate time that needs to be measured experimentally. Nevertheless, it is probable that by adding the viral organism the needs for the bacteria's food increase and the bacteria's production diminished. So, the optimum bacteria production needs to be calculated based on the volume of bacteriophages added and the concentration of these viral organisms wanted. Also, the rate and concentrations of the culture medium that is added to the reactor after the infection of the bacteriophage needs to be measured experimentally.

9. Filtration

After the production of phages comes the separation process that consists in ultracentrifugation. Which generates a centrifuge force that separates the macromolecules of the mixing according to its different sizes. The velocity that the molecules acquire during the centrifugation called sedimentation velocity and it determines the final location of the molecule after this process (Fields, 2019). This methodology allows the separation between the phages from the bacterium and unwanted matter. Additionally, it is a common process; its machinery can found at different prices and models, which allows a greater economic accessibility.

CONCLUTIONS

Rational design, based on phagemids, represented a personal challenge for all team members. Filtering, organizing and selecting the most viable antigenic targets required advanced knowledge in computer science, biochemistry, genetics and virology. Each of these branches of science helped to better prioritize resources for design, as well as test the in silico construction of a functional phagemid genome. Also, as immunoinformatics processes were included, the total library of useful peptides was reduced. Thanks to each of the knowledge applied and the free digital tools used, it was possible to propose a vaccine design against the SARS-CoV-2 spike protein.

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