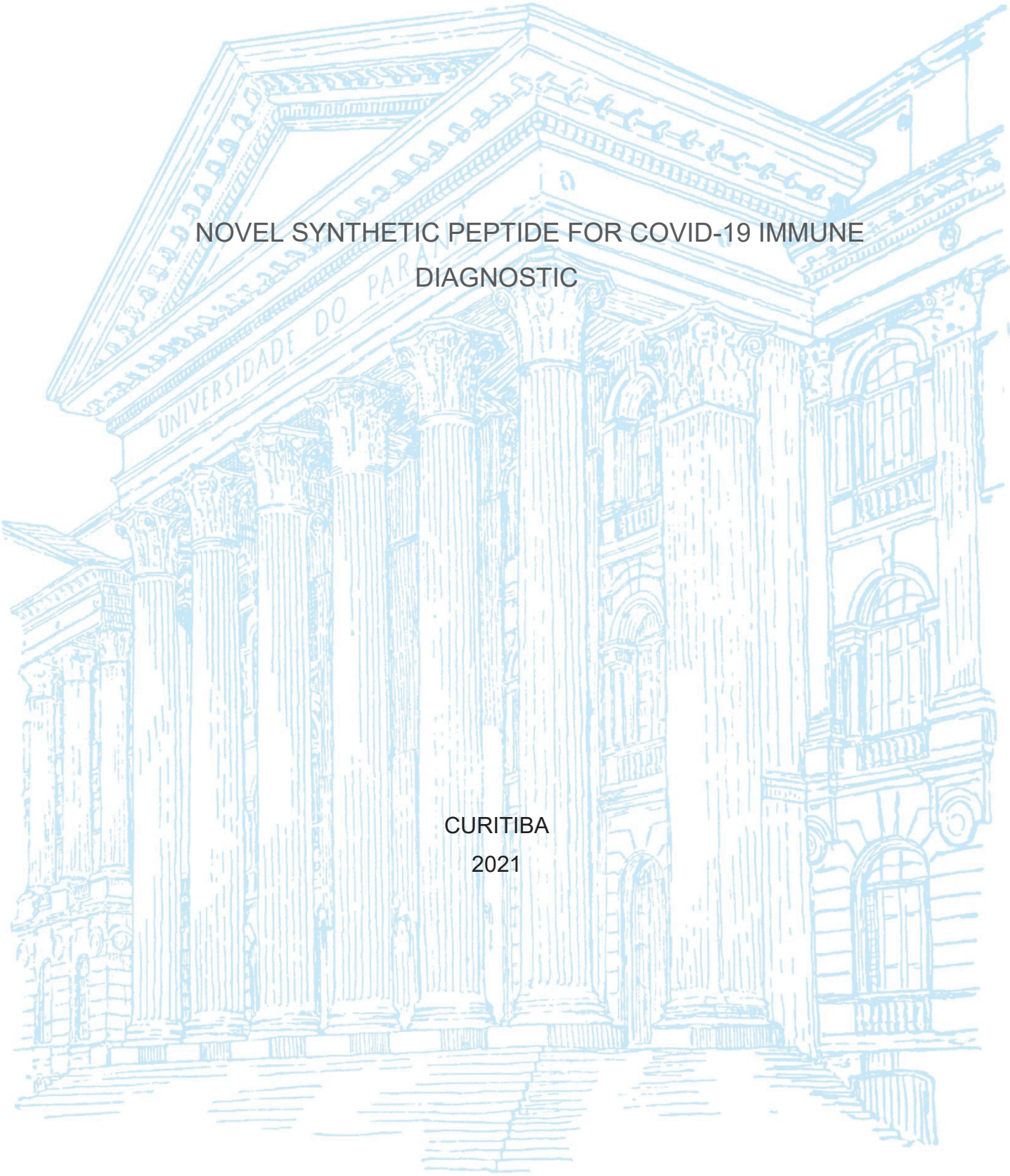
UNIVERSIDADE FEDERAL DO PARANÁ

MANUEL HOSPINAL SANTIANI



NOVEL SYNTHETIC PEPTIDE FOR COVID-19 IMMUNE DIAGNOSTIC

CURITIBA

2021

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NOVEL SYNTHETIC PEPTIDE FOR COVID-19 IMMUNE DIAGNOSTIC

Tese apresentada ao curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, como requisito parcial à obtenção do título de em Engenharia de Bioprocessos e Biotecnologia.

Orientadora: Profa. Dra. Vanete Thomaz Soccol

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **MANUEL HOSPINAL SANTIANI** intitulada: **NOVEL SYNTHETIC PEPTIDE FOR COVID-19 IMMUNE DIAGNOSTIC**, sob orientação da Profa. Dra. VANETE THOMAZ SOCCOL, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 01 de Outubro de 2021.

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VANETE THOMAZ SOCCOL
Presidente da Banca Examinadora

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RESUMO

Em dezembro de 2019, um novo coronavírus, denominado síndrome respiratória aguda grave coronavírus 2 (SARS-CoV-2), foi identificado como a causa de pneumonia com graves problemas respiratórios e o surto em Wuhan, China. A rápida propagação global do vírus SARS-CoV-2 resultou na pandemia do coronavírus 2019 (COVID-19) que resultou em quase 4,5 milhões de mortes desde o início da pandemia até setembro do 2021. No início e durante a os primeiros meses da pandemia, o desenvolvimento de métodos de diagnóstico assumiu grande importância, uma vez que permitiriam a identificação de indivíduos infectados e o acompanhamento da propagação da pandemia, que continuou com o surgimento de novas linhagens. Para resolver este problema, no presente trabalho, dois peptídeos sintéticos da proteína Spike foram identificados por sobreposição em abordagens in silico e in vitro. Assim, foram sintetizados quimicamente e utilizados como antigénios para testes ELISA indirectos. Foram utilizados desenhos experimentais para optimizar os ensaios e identificar a influência de cada variável. A base de testes ELISA indirectos em Peptídeos P.SC2.S.203 foi capaz de diferenciar entre amostras positivas e negativas e alcançou 91,67% de sensibilidade e 100% de especificidade para o diagnóstico serológico de IgM e para a sensibilidade serológica de IgG 91,67% e 100% de especificidade o peptídeo seleccionado aplicado ao ELISA como método de rastreio para o diagnóstico de COVID 19 representa uma ferramenta potencial para ajudar a combater a doença.

Palavras-chave: 1. teste indirecto ELISA, 2. COVID-19. 3. Peptídeos sintéticos.

ABSTRACT

In December 2019, a novel coronavirus, termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was identified as the cause of pneumonia with severe respiratory distress and outbreaks in Wuhan, China. The rapid and global spread of SARS-CoV-2 resulted in the coronavirus 2019 (COVID-19) pandemic which resulted in nearly 4.5 million deaths since the beginning of the pandemic. Earlier during the pandemic, the development of diagnostic methods took on great importance because they would allow the identification of individuals and the tracking of the spread of the pandemic which continued with the emergence of new lineages. To address this problem, in the present work, two synthetic peptides from Spike protein were identified by overlapping in silico and in vitro approaches. Thus, they were chemically synthesized and used as antigens for indirect ELISA tests. Experimental designs were used to optimize the assays and identify the influence of each variable. Indirect ELISA test base in Peptides P.SC2.S.203 was able to differentiate between positive and negative samples and achieved 91,67% sensitivity and 100% specificity for serological IgM diagnostic and for serological IgG 91,67% sensitivity and 100% specificity the selected peptide applied to the ELISA as a screening method for the diagnosis of COVID 19 represents a potential tool to help combat the disease.

Keywords: 1. indirect ELISA tests, 2. COVID-19, 3. synthetic peptides

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LIST OF ABBREVIATIONS OR ACRONYMS

DNA	-Deoxyribonucleic acid
RNA	-Ribonucleic acid
WHO	-World Health Organization
qPCR	-Real-time polymerase chain reaction
PCR	-Polymerase chain reaction
aa	-Amino acid
SARS-CoV-2	-Severe acute respiratory syndrome coronavirus 2
SD	-Standard deviation

SYMBOL LIST

©	-Copyright
µL	-Microliters
®	-Registered trademark
µm	-Micrometers
%	-Percentage
ºC	-Celsius grade
pb	-Base pairs
mg	-Milligram
mM	-Millimolar

SUMÁRIO

1. OBJECTIVE

1.1 **GENERAL OBJECTIVE**

The aim of this study was to develop a diagnostic of COVID-19 by the used of peptides in test of ELISA.

1.2 SPECIFIC OBJETIVE

- Selection peptides for diagnostic COVID-19;
- Spot synthesize the protein selected and immunodetect the reactive spots;
- *In silico* evaluate the immunogenicity properties of peptides;
- Evaluate peptides' reactivity against human anti-SARS-CoV-2 IgG and IgM antibodies;
- Development ELISA test using peptides,
- Evaluate the performance of ELISA test for identification anti-SARS-CoV-2 antibodies.

2. LITERATURE REVIEW

2.1 ETIOLOGICAL AGENT

Coronaviruses (CoV) are a highly diverse family of single-stranded, positive-sense RNA viruses that use a DNA replicase enzyme (or a dependent RNA polymerase) in their replication. They infect a wide group of species, including humans, birds, companion animals, and cattle, which can be reservoirs of the virus. CoV are of the order Nidovirales, suborder Coronavirineae, family Coronaviridae, subfamily Orthocoronavirinae, which are subdivided into four genera: alphacoronavirus, betacoronavirus, gamma-coronavirus, and deltacoronavirus. The betacoronavirus genus exclusively infects mammalian species, and where the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), SARS-CoV, and MERS-CoV are found, they are highly pathogenic and of zoonotic origin. Human and animal coronavirus infections result mainly in respiratory and enteric diseases (CORONAVIRIDAE STUDY GROUP OF THE INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES, 2020).

COVID-19 (coronavirus disease 2019) emerged in December 2019 in China and spread rapidly around the world causing a serious global public health problem (ROTHAN; BYRAREDDY, 2020; WORLD HEALTH ORGANIZATION (WHO), 2020). COVID-19 is a disease caused by the SARS-CoV-2, which primarily targets the respiratory system and can cause an acute respiratory condition characterized by at least two of the following symptoms: the most common symptoms are fever (83-98%), followed by fatigue (70%) and dry cough (59%). However, severe cases of infection progress to pneumonia, severe acute respiratory syndrome, renal failure, and acute respiratory distress syndrome acute respiratory distress syndrome, and is potentially fatal (GUAN et al., 2020). in addition, you have other symptomatology describes as “long COVID” illness in persons who have either recovered from covid-19 but continue to report lasting effects after infection which is characterized by fatigue, dyspnea, and loss of taste or smell (RAVEENDRAN; JAYADEVAN; SASHIDHARAN, 2021; SYKES et al., 2021).

2.2 SARS-COV-2

The viral RNA of SARS-CoV-2 encodes four structural proteins: small envelope (E); matrix (M), Nucleocapside (N) spike glycoprotein (S); seven accessory proteins (Orf3a, Orf6, Orf7a, Orf7b, Orf8, Orf9, Orf10) and an open reading frame polyprotein 1a and 1b, encoded at Orf1ab, which can be proteolytically cleaved into 16 putative nonstructural proteins (Nsp), which may be involved in viral RNA replication, transcription and supporting role (CHAN et al., 2020a). The E and M proteins are important in viral assembly in coronaviruses. The N protein forms complexes with genomic RNA and is important to increase the efficiency of transcription and viral assembly (WANG, HONGYE *et al.*, 2020).

During the viral infection, the virus can inject its genome into the host cell through two pathways, via endosomes or direct fusion of the viral envelope with the host cell membrane, S protein binds to the angiotensin-converting enzyme receptor 2 (ACE2) of host cells, that is proteolytically processed into S1 and S2 subunits that remain associated, where conformational rearrangements occur that cause shedding of S1, and cleavage of S2 by host cell proteases, and exposure of a fusion peptide adjacent to the proteolysis site of S2 (CHAN et al., 2020b). The next stage is the viral gRNA entry into the host cell cytoplasm and translated by the host ribosomes. The translation products, polyprotein pp1ab, are proteolytically cleaved into nonstructural proteins Nsp1 − 16 by the viral proteases, along with other factors. Nsp12-16 provide the required enzymatic function for viral genome replication/transcription inside the replication-transcription complex (ROMANO et al., 2020). The RNA (+) strand first gets replicated to the RNA (-) strand and then the negative-strand is used either for replication to the RNA (+) or transcription of sub-genomic mRNAs. These sub-genomic mRNAs are translated to S, M, E, N, and the accessory proteins. The S, M, and E proteins enter the endoplasmic reticulum (ER), and the N protein attaches to the genomic RNA (+) strand to produce nucleoprotein complex. The complex and the structural proteins move to the ER-Golgi where the packaging, mature of the virus will take place, and bud off from the Golgi in the form of small vesicles. These vesicles travel to the host cell membrane where they are released

into the extracellular region through exocytosis to continue the process of virus infection and propagation (ARYA et al., 2020).

The transmission of the virus between humans is similar to the mechanism of other viruses that attack the respiratory system. The principal mechanism of transmission of is via infected respiratory droplets, with viral infection occurring by direct or indirect contact respiratory tract, when deposition occurs in the mucous membranes are also susceptible to being infected in the membranes of conjunctiva and gastrointestinal tracts (CEVIK et al., 2020). Transmission risk depends on socioeconomic factor, environment, infected individual's condition (CEVIK et al., 2021).

There are currently more than 1,352,250 publicly available complete or near-complete genome sequences of SARS-CoV-2 and the number continues to grow. To follow the constant increase of the virus genomes, a dynamic nomenclature system was created to identify the variants, that arises from a set of fundamental evolutionary and phylogenetic principles, these are called variant and lineages. variant are viruses genome that contains a particular set of mutation (substitution, deletion or/and insertion), lineages is a collection of mutations who are descendants of a branch of a phylogenetic tree, within a lineage, there may be additional mutations which revert some changes or accumulate new ones (RAMBAUT et al., 2020). The nomenclature is based on the methodology on PANGO lineages (Phylogenetic Assignment of Named Global Outbreak). Actually 1552 variant have been reported and 19 lineages (as of 10 September 2021) (table 1)

Table 1. Summary of currently defined lineages adapted from CoV-lineages.org (source: author).

Label	Description	Pango lineages representative	Tags	Mutation sites
A.23.1-like	A.23.1 lineage defining mutations	A.23.1	A.23.1	nsp3:L741F, nsp6:M86I, nsp6:L98F, nsp6:M183I, S:R102I, S:F157L, S:V367F, S:Q613H, S:P681R, ORF8:L84S, ORF8:E92K, N:S202N
AV.1-like	AV.1 lineage defining mutations	AV.1	AV.1	S:D80G, S:T95I, S:G142D, S:Y144-, S:N439K, S:E484K, S:D614G, S:P681H, S:I1130V, S:D1139H, M:A63T, M:H125Y, N:I157V, N:R203K, N:G204R
B.1.1.318-like	Defining of lineage B.1.1.318	B.1.1.318	B.1.1.318, VUI-21FEB-04, VUI202102/04	nuc:C3961T, nsp3:K1693N, nsp4:T173I, nsp4:A446V, nsp5:T21I, del:11287:9, nsp15:V320M, S:T95I, del:21990:3, S:E484K, nuc:T23287C, S:P681H, S:D796H, nuc:C25276A, M:I82T, del:27887:15, ORF8:E106*, nuc:A28271G, del:28895:3
B.1.1.7-like+E484K	This is a variant of cB.1.1.7 that additionally contains E484K.	B.1.1.7	B.1.1.7, E484K	nuc:C913T, ORF1ab:T1001I, ORF1ab:A1708D, nuc:C5986T, ORF1ab:I2230T, ORF1ab:SGF3675-, nuc:C14676T, nuc:C15279T, nuc:T16176C, s:HV69-, s:Y144-, S:E484K, s:N501Y, s:A570D, s:P681H, s:T716I, s:S982A, s:D1118H, nuc:C26801T, 8:Q27*, 8:R52I, 8:Y73C, N:D3L, N:S235F

Alpha (B.1.1.7-like)	B.1.1.7 lineage defining mutations	B.1.1.7 sublineages Q.1, Q.2, Q.3, Q.4, Q.5, Q.6, Q.7, Q.8	B.1.1.7, VOC 202012/01	nuc:C913T, ORF1ab:T1001I, ORF1ab:A1708D, nuc:C5986T, ORF1ab:I2230T, ORF1ab:SGF3675-, nuc:C14676T, nuc:C15279T, nuc:T16176C, s:HV69-, s:Y144-, s:N501Y, s:A570D, s:P681H, s:T716I, s:S982A, s:D1118H, nuc:C26801T, 8:Q27*, 8:R52I, 8:Y73C, N:D3L, N:S235F
Beta (B.1.351-like)	Defining of lineage B.1.351	B.1.351 Sublineages: B.1.351.1, B.1.351.2, B.1.351.3, B.1.351.4	B.1.351, VOC-20DEC-02, VOC202012/02, Beta, 501.V2, 20H, GH	NSP2:T85I, ORFORF1ab:K1655N, ORFORF1ab:K3353R, S:D80A, S:D215G, S:E484K, S:N501Y, S:A701V, ORF3a:Q57H, ORF3a:S171L, E:P71L, N:T205I, del:22280:9, del:11287:9
Epsilon (B.1.427/429-like)	Defining of lineage B.1.427	B.1.427, B.1.429	B.1.427, B.1.429, Eta	S:S13I, S:W152C, S:L452R, S:D614G, ORF1a:I4205V, ORF1b:D1183Y, nuc:C2395T, nuc:T2597C, nuc:T24349C, nuc:G27890T, nuc:A28272T
Eta (B.1.525-like)	This variant is a cluster of E484K containing genomes	B.1.525	B.1.525, VUI-21FEB-03, VUI202102/03, Eta, G/484K.V3, 20A	nuc:C1498T, nuc:A1807G, nuc:T8593C, nuc:C9565T, nsp12:P323F, nuc:C18171T, nuc:A20724G, S:Q52R, S:E484K, S:Q677H, S:F888L, nuc:C24748T, E:L21F, M:I82T, nuc:A28699G, nuc:G29543T
Kappa (B.1.617.1-like)	Defining constellation for lineage B.1.617.1	B.1.617.1	B.1.617.1, VUI-21APR-01	nuc:C3457T, nsp3:T749I, nsp6:T77A, nsp13:M429I, nsp15:K259R, S:L452R, S:E484Q, S:P681R, ORF3a:S26L, ORF7a:V82A, N:R203M
Delta (B.1.617.2-like) +K417N	Defining constellation for lineage B.1.617.2 with the addition of S K417N	B.1.617.2 Sublineages: AY.1, AY.2, AY.3, AY.3.1, AY.4, AY.5, AY.5.1, AY.5.2, AY.6, AY.7, AY.7.1, AY.7.2, AY.8, AY.9, AY.10, AY.11, AY.12, AY.13, AY.14, AY.15, AY.16, AY.17, AY.18, AY.19, AY.20, AY.21, AY.22, AY.23, AY.24, AY.25, AY.26, AY.27, AY.28, AY.29, AY.30, AY.31	B.1.617.2, K417N, VOC-21APR-02, VUI-21APR-02	S:T19R, S:K417N, S:L452R, S:T478K, S:P681R, S:D950N, ORF3a:S26L, M:I82T, ORF7a:V82A, ORF7a:T120I, N:D63G, N:R203M, N:D377Y
Delta (B.1.617.2-like)	Defining constellation for lineage B.1.617.2	B.1.617.2, AY.1, AY.2	B.1.617.2, VOC-21APR-02, Delta, VUI-21APR-02	S:T19R, S:G142D, S:L452R, S:T478K, S:P681R, S:D950N, ORF3a:S26L, M:I82T, ORF7a:V82A, ORF7a:T120I, N:D63G, N:R203M, N:D377Y
Label	**Description**	**Pango lineages representative**	**Tags**	**Mutation sites**
B.1.617.3-like	Defining constellation for lineage B.1.617.3	B.1.617.3	B.1.617.3, VUI-21APR-03	nuc:C835T, nsp3:A1526V, nsp3:T1830I, nsp5:A194S, nsp6:A117V, nuc:C16293T, S:T19R, S:L452R, S:E484Q, S:P681R, S:D950N, nuc:T27384C, ORF7a:V82A, ORF8:T26I, N:P67S, N:R203M, N:D377Y
Theta (P.3-like)	Defining constellation for lineage P.3	P.3	P.3, VUI-21MAR-02, VUI202103/02	nsp3:D736G, nsp4:L438P, nsp6:D112E, nsp7:L71F, nsp13:A368V, del:21980:9, S:E484K, S:N501Y, S:P681H, S:E1092K, S:H1101Y, S:V1176F, ORF8:K2Q, nuc:C7564A, nuc:C12049T, nuc:T23341C, nuc:T24187A
Gamma (P.1-like)	Defining constellation for lineage P.1	P.1 Sublineages: P.1.1, P.1.2, P.1.3, P.1.4, P.1.5, P.1.6, P.1.7, P.1.8, P.1.9, P.1.10, P.1.10.1, P.1.10.2, P.1.11	P.1, VOC-21JAN-02, VOC202101/02	nuc:T733C, nuc:C2749T, ORFORF1ab:S1188L, ORFORF1ab:K1795Q, del:11287:9, nuc:C12778T, nuc:C13860T, S:T20N, S:P26S, S:K417T, S:E484K, S:N501Y, S:T1027I, ORF8:E92K, nuc:28262+AACA, N:P80R
Zeta (P.2-like)	Defining constellation for lineage P.2	P.2	P.2, VUI-21JAN-01, VUI202101/01	ORFORF1ab:L205V, nuc:C11824T, S:E484K, nuc:A12964G, nuc:C28253T, N:A119S, N:M234I
Lambda (C.37-like)	C.37 lineage defining mutations	C.37	Lambda, C.37	nuc:T1246I, ORF1a:P2287S, ORF1a:F2387V, ORF1a:L3201P, ORF1a:T3255I, ORF1a:G3278S, ORF1a:SGF3675-, ORF1b:P314L, S:G75V, S:T76I, S:RSYLTPG246-, S:L452Q, S:F490S, S:D614G, S:T859N, N:P13L, N:R203K, N:G204R, N:G214C
Iota (B.1.526-like)		B.1.526 Sublineages: B.1.526.1, B.1.526.2, B.1.526.3	B.1.526, Iota	nuc:C3037T, ORF1a:T265I, ORF1a:L3201P, ORF1ab:SGF3675-, ORF1b:P314L, ORF1b:Q1011H, nuc:A20262G, S:L5F, S:T95I, S:D253G, S:E484K, S:D614G, S:A701V, ORF3a:P42L, ORF3a:Q57H, N:P199L, N:M234I,

				ORF8:T11I, S:S477N, S:Q957R, N:P13L, N:S202R, nuc:A28271-
A.23.1-like+E484K	This is a variant of A.23.1 that additionally contains E484K.	A.23.1	A.23.1, VUI-21FEB-01, VUI202102/01	nsp3:L741F, nsp6:M86I, nsp6:L98F, nsp6:M183I, S:R102I, S:F157L, S:V367F, S:E484K, S:Q613H, S:P681R, ORF8:L84S, ORF8:E92K, N:S202N
Mu (B.1.621-like)		B.1.621	B.1.621	nuc:C3037T, ORF1a:T1055A, ORF1a:T1538I, nuc:C6037T, ORF1a:T3255I, ORF1a:Q3729R, nuc:A13057T, ORF1b:P314L, ORF1b:P1342S, nuc:C18877T, nuc:T19035C, nuc:C20148T, S:T95I, S:Y145N, S:R346K, S:E484K, S:N501Y, S:D614G, S:P681H, S:D950N, ORF3a:Q57H, nuc:A26492T, ORF8:T11K, ORF8:P38S, ORF8:S67F, nuc:A28272T, N:T205I

2.2.1 S protein

The homo-trimeric S protein plays a role in receptor recognition and binding while the membrane-anchored in the infection phase, which makes this protein a target for the development of diagnostic kits, effective vaccines and therapeutics to help arrest the spread of the COVID-19 pandemic. The total length of S protein is 1273 amino acid (aa), and consists of a signal peptide (SP) (1–13 residue) located at the N-terminus, and two subunits (S1 and S2). The S1 subunit (14–685 residues), there is an N-terminal domain (14–305 residues) and a receptor-binding domain (RBD, 319–541 residues), there is a furin proteolysis sites (FPS) FPS1 (681-685 residues); the S2 subunit (686–1273 residues, the fusion peptide (FP) domain (788–806 residues), FPS2 (811-815 residues), two heptad repeat (HR) domain sequence 1 (HR1) (912–984 residues), HR2 (1163–1213 residues), transmembrane (TM) domain (1213–1237 residues), and cytoplasm domain (1237–1273 residues) (HUANG et al., 2020; XIA, 2021) (**Fig. 1**).

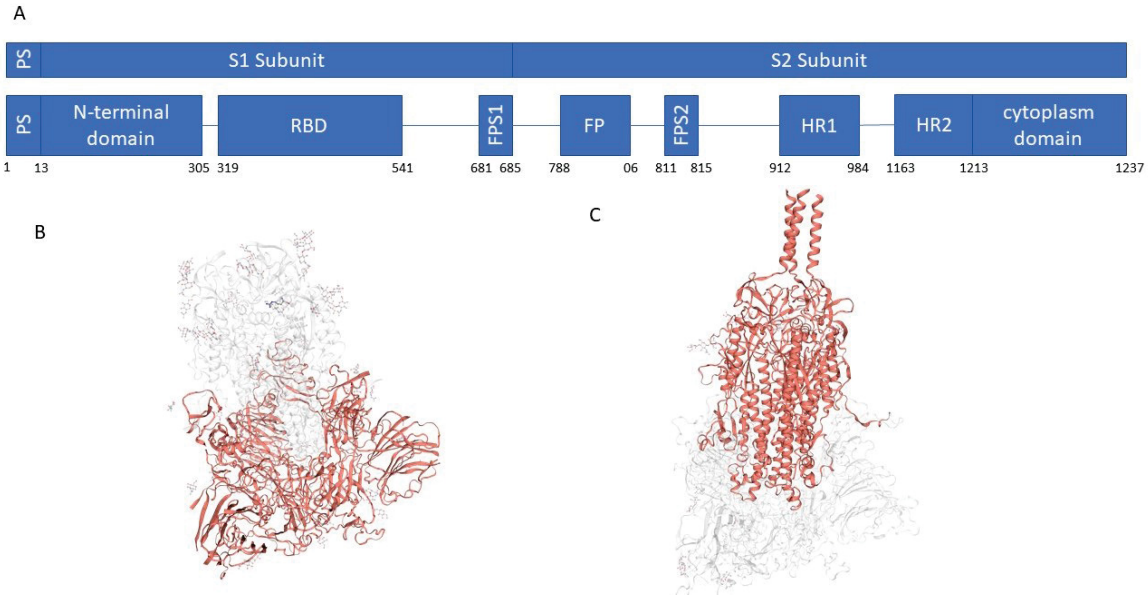

Fig. 1. Structure of the SARS-CoV-2 S protein A. Schematic representation of the SARS-CoV-2 spike. B. The S protein RBD closed, in red shows S1 subunit. C. The S protein RBD closed, in red shows S2 subunit (source: author)..

While SARS-CoV-2 has a lower mutation rate than other viruses due to proof-reading mechanisms (CALLAWAY, 2020), so far, 3540 variants have been reported in protein S, with an average of 3 mutations in each amino acid of the protein, having 0 to 9 variants in a single amino acid (POKHREL et al., 2021). Fig. 2 shows the main mutations that define a lineage in function of protein S, showing the frequency with which, they can be found and the comparison between different lineages.

Fig. 2. The comparative frequency of the mutations presents in SARS-CoV-2 S protein by lineage dates from Outbreak (available at https://outbreak.info/compare-lineages?pango=Delta&pango=Alpha&pango=Beta&pango=Gamma&pango=Zeta&pa

ngo=Lambda&pango=Epsilon&pango=Theta&pango=B.1.617.2&pango=Mu&gene=S&threshold=75&dark=true). Accessed 10 September 2021.

2.3 DIAGNOSTIC

The diagnosis of COVID-19 is initially based on the clinical manifestations of patients infected with SARS-CoV-2. However, these are insufficient for a definitive diagnosis since the symptoms and signs of COVID-19 infection are similar to those of many other common febrile and respiratory diseases. To have a final diagnosis, auxiliary tests are required, the most common being molecular and serological tests. Reliable diagnostics are critical for detecting cases of COVID-19, interrupting transmission, understanding epidemiology, monitoring drug efficacy, and the impact of social and public health measures (WHO - R&D BLUEPRINT, 2020). The identification of active cases allows the early isolation of patients, preventing further spread of the disease, and consequently reducing the number of cases and deaths. In this sense, mass testing is an essential tool for pandemic control, since it allows the identification of infected individuals, and enables the authorities to map the regions with outbreaks of the disease, and thus take measures to contain its spread (LEE; YEO; NA, 2020).

Since the beginning of the pandemic, more than 225 million cases have been confirmed, with 4.64 million deaths worldwide. Brazil ranks third in the number of confirmed cases (20.9 million) and second in the number of deaths (0.58 million), and some 57.1 million diagnostic tests have been performed (Worldometer. Worldometer's COVID-19 data. 2021. Available at: https://www.worldometers.info/coronavirus/. Accessed on: 10/09/2021).

2.3.1 Molecular tests

Molecular tests are those that allow the causative agent of the disease to be identified by the detection of the genetic material. The main test of this category, used in the diagnosis of COVID-19, is the "reverse transcriptase real time polymerase chain reaction" (RT-qPCR). The sensitivity of this technique is influenced by the type of biological sample with which the analysis is undertaken. By estimation, the frequency of negative results when patients are tested only once has reached 30-50% in real cases of COVID-19, and a large number of "suspected" cases with clinical features typical of COVID-19 (WANG et al., 2020b). In addition, the period of 2 to 3 weeks after symptom onset, the viral load

begins to decrease, making the process of isolation of genetic material difficult due to the low load, which can generate false negative results. Other possible sources of error in this technique would be found in the way the sample is collected. This step is of great importance for any methodology based on the detection of genetic material, any error at this stage would generate an increase in false positive or false negative results (PASCARELLA et al., 2020). PCR-based methodologies sensitivity is reflected in the care taken in sample collection, isolation of the genetic material and execution of the assay. Another point to take into account is the need for expensive equipment or reagents.

2.3.2 Image diagnosis

A method indicative of the disease is by computed tomography (CT) scan of the chest, employed mainly in symptomatic patients. The technique identifies opaque areas in the lungs and is employed in conjunction with other methods after symptoms are present. Although it does not specifically diagnose virus infection, CT is important to determine the severity of infection in symptomatic patients with the disease, and to follow its progression. However, its sensitivity varies depending on the stages of infection and the patient's symptoms (BERNHEIM et al., 2020; ZARIFIAN et al., 2021).

2.3.3 Serological tests

Serological tests detect the presence of specific antigen from SARS-CoV-2 or specific antibodies against the pathogen by measuring antibody responses against pathogens in body fluids, especially in blood, serum, saliva and other samples (LI et al., 2020) . Some well-established techniques are enzyme-linked immunosorbent assay (ELISA), immunochromatography (or lateral flow), immunoelectrochemistry. Compared to PCR-based methodologies, serological tests are advantageous with faster turnaround time, high throughput, less workload, diagnosis of infection, the measurement of protective antibodies after vaccination and also the assessment of the prevalence of immunity in a population (SETHURAMAN et al., 2020).

Laboratory and clinical diagnosis should occur simultaneously to give faster results. PCR diagnostic techniques are more costly and less accessible when compared to serological tests in. In addition, these techniques have

limitations for use in settings where large-scale testing (mass testing) including asymptomatic cases, in the context low and lower-middle income countries, which are at a disadvantage in relation to high-income countries in terms of access to reagents and equipment. In these situation serological tests, mainly evaluating IgG antibodies, stand out due to the possibility of testing over a long post-symptomatic period or even in asymptomatic cases. The most explored option for serological tests has been immunochromatographic tests because they are of the point-of-care type, allowing quick results with practicality. However, they can have high variability in specificity and sensitivity, leading to false-negative diagnoses. On the other hand, serological tests by ELISA technique overcome the limitations presented by other techniques when applied in these situations, besides being a well-established technique, well-diffused among diagnostic laboratories and subject to automation. For these reasons, in the present work we will focus on the development of a diagnostic kit for covid using serological approach.

Antigen tests

The objective of these tests is the detection of viral particles in samples, for which they use capture monoclonal antibodies targets being usually protein S or N of the SARS-CoV-2. (DIAO et al., 2020; SHERIDAN, 2020). Also, these antibodies must be labeled with a fluorescent probe (FLA) or chemiluminescent substances (CLIA).

Clinical samples are mainly nasal and nasopharyngeal swab. The sampling process is crucial because the detection of viral antigen requires a minimal antigen load on the samples (YAMAYOSHI et al., 2020).

The advantages of these tests are the speed of test execution around 15 min., no complex laboratory facilities are required can be a point-of-care, are relatively inexpensive. On the other hands, they require a higher viral load, low sensitivity. (GUGLIELMI, 2021; YAMAYOSHI et al., 2020).

Antibody tests

These tests are designed for the detection of immunoglobulin (Ig) against antigens of the pathogen and are important for determining immune individuals, people with low viral dose showing false-negative RT-qPCR, and patients with late notification. SARS-CoV-2 induces humoral and cellular immune responses.

The diagnosis of COVID-19 by targeting the humoral immune response may be advantageous from several perspectives, among them not requiring a high biosafety level laboratory. Antibody tests provide evidence about the serum prevalence of a population and allow identifying regions with a higher incidence of the disease (JIA; LU, 2020). Moreover, they are particularly important for identification of convalescent, asymptomatic and mild cases that might have been missed by other surveillance methods (YONG et al., 2020). In addition, they permit the identification of immune response patterns and also can be used to monitor the population seroconversion after vaccination (KRAMMER; SIMON, 2020).

Enzyme-Linked Immunosorbent Assay

ELISA was invented in the 1960s and is widely used from the 1970s till now, is the most commonly serological test, with an average detection time between 2 and 8 h (MEKONNEN et al., 2021). ELISA tests can be classified according to the detection strategy, indirect ELISA, the sandwich ELISA, double antibody ELISA, competitive ELISA, blocking ELISA, and other different types (BUTLER, 2000).

The indirect ELISA method is most commonly used in COVID-19 serological diagnosis. This method takes as a base the virus protein (N, S), S protein subunit (S1, S2), or protein domain (RBD), which will be immobilized in a solid carrier, which will be exposed to individuals' serum for the generation of the antigen-antibody complex, is detected through the use of a secondary antibody to produce a chromogenic reaction (MEKONNEN et al., 2021). at present, there are several commercial SARS-CoV-2 diagnostic kits available those that use mainly recombinant proteins for antibody detection (VEDOVA-COSTA et al., 2021).

2.3.4 Antigen for serological test

A wide variety of molecules can be used in serological tests, such as proteins, peptides, deoxyribonucleic acids (DNAs), ribonucleic acids (RNAs), antibiotics, hormones drugs, among others. Which directly reflect on the specificity achieved by the test (HOSSEINI et al., 2018).

The identification and choice of an antigen are key component to develop a serological test. For diagnostics of COVID-19, using the ELISA methodology

recombinant antigens from two viral proteins, S and N stand out for the development of tests detecting IgM and IgG antibodies (LIU et al., 2020). With the use of protein S showing the highest sensitivity (KONTOU et al., 2020; ONG et al., 2020). The production and use of proteins as antigens for diagnostic tests is frequently hindered by variation in species and leads to high costs for purification (GOMARA; HARO, 2007). An alternative to recombinant proteins are synthetic peptides, which have been explored as antigens for the serological diagnosis of several diseases, as they are able to present high antigenicity, high antigen-antibody affinity and specificity. (VAN REGENMORTEL, 2001).

These peptides can be identified by two strategies. The first approach is the *in vitro* analysis of the peptides by biotechnological and biochemical tools such as SPOT-synthesis techniques. SPOT-synthesis or synthesis on solid support using amino acids with protecting groups, such as fluorenylmethyloxycarbonyl (Fmoc) (HILPERT; WINKLER; HANCOCK, 2007a). Its synthesis can be performed in automated equipment, providing reproducibility and agility. This approach has been explored in different proteomics or immunological studies, where it was used to identify immunogenic regions of great potential for use in vaccines development for different viruses. This could also be applied to the development of serological diagnostic tests, especially in the identification of antibodies against SARS-CoV-2 type IgM and IgG (CHAN et al., 2020b; JIANG et al., 2020; WANG et al., 2020a).

The second approach in silico prediction techniques represent a powerful approach which allows us to screening proteins to identify potential immunogenic regions. We will focus on the prediction of B-cell epitopes, which are a key step in development Serological tests. Several computational methods for B-cell epitope prediction have been developed (EL-MANZALAWY; HONAVAR, 2010; RAOUFI et al., 2020). However, the prediction algorithms performance is far from ideal, but as new computational tools (e.g. machine learning) emerge, they are becoming more and more sophisticated. BepiPred, ABCpred, LBtope, BCPREDS, NeoClone are methods based on machine learning. B-cell epitopes prediction is not only limited to the above mentioned tools, but it is also necessary to analyze other factors, that includes: antigenicity, surface accessibility prediction, hydrophilicity prediction, and other (SANCHEZ-TRINCADO; GOMEZ-PEROSANZ; RECHE, 2017).

Based on that, this work proposes the investigation of new active peptides derived from S protein of SARS-CoV-2. Using two strategies for identified immunogenic regions and development a serological test with high sensibility and specificity.

3. MATERIAL AND METHODS

The methodology of this work comprehends two major steps. The first one was divided in process of peptides selection, synthesis and evaluation. The second was development and validation an immune assay for diagnosis for COVID19.

3.1 MATERIAL

3.2 SERUM

This retrospective study included 38 serum from adult and healthy volunteers collected before the pandemic began. Serum samples (n = 40) were obtained from COVID-19 patients confirmed to at least two conventional laboratory test for SARS-CoV-2, Lateral Flow, RT-qPCR in May 2020.

3.3 PROTEIN SPOT-SYNTHETIZED PEPTIDES AND IMMUNODETECTION ASSAY

To select and evaluate reactive peptides from S protein (QIG55994.1) their sequences were spot-synthesized by overlapping peptides (15 residues) offset of four amino acids scanning. The Spot synthesis was performed on a cellulose membrane with Fmoc-protection using an automated spot peptide synthetizer (Intavis Bioanalytical Instruments, Nattermannallee, Germany) (HILPERT; WINKLER; HANCOCK, 2007a).

For immunodetection assay, the cellulose membrane was firstly blocked, for nonspecific binding, by overnight agitation with 3% (w/v) casein and 0.5% (w/v) sucrose dissolved in TBS-T (0.1% Tween 20 (v/v) in TBS) at 4°C. Afterwards, the membrane was washed with TBS-T 0.1% for 10 min under agitation, and probed, for 90 min at 37°C, with patients' serum or negative control diluted 1:100 in blocking buffer (3% (w/v) casein, 0.5% (w/v) sucrose and TBS-T 0.1%). The membrane was washed again and incubated with biotin-labeled secondary antibody (1:40,000) diluted in blocking buffer for 60 min at 37°C, followed by an incubation step with Streptavidin (1:10,000) diluted in blocking buffer for 60 min at 37°C. After two washes, positive spots were visualizing by ECL™ system. To analyze and compared the generated images we used the imageJ software V.1.52n (SCHNEIDER; RASBAND; ELICEIRI, 2012) with the plugin Protein array Analyzer are available on

http://image.bio.methods.free.fr/ImageJ/?Protein-Array-Analyzer-for-ImageJ&lang=fr.

Membranes were evaluated for IgG. A regeneration process was carried out and checked for the membrane before using different antibodies, to ensure the removal of primary and secondary antibodies, and thus avoiding false positives during the assays.

The regeneration of the membrane was carried out in two steps. In the first step the membranes were treated with the regeneration buffer I (solution 8 M of urea with 1% SDS, 2 times for 30 min). Then, they were treated with regeneration buffer II (solution of 100 mL of acetic acid, 500 mL of Ethanol and 400 mL of water) for 30 min with a light agitation both at room temperature. Finally, they were washed at least five times with methanol and dried on air, the membrane was storage at -80 ºC.

3.4 PEPTIDE SELECTION BY BIOINFORMATIC

The reference genome used throughout this study is from the severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1 (GenBank: MT126808.1) where we obtained S protein sequences (QIG55994.1), with these sequence we proceeded to search for antigenic regions using the following tools: Bepipred Linear Epitope Prediction 2.0, Surface, Accessibility, Antigenicity, Hydrophilicity available on BcePred (https://webs.iiitd.edu.in/raghava/bcepred) (SAHA; RAGHAVA, 2004) , Kolaskar & Tongaonkar Antigenicity (KOLASKAR; TONGAONKAR, 1990), Bebipred 2.0 (JESPERSEN et al., 2017), Both prediction algorithms are available on the IEDB B cell prediction tool page IEDB Analysis Resource (http://tools.iedb.org/main/bcell) (FLERI et al., 2017; KIM et al., 2012; ZHANG et al., 2008), ABCpred (webs.iiitd.edu.in/raghava/abcpred/) (EL-MANZALAWY; DOBBS; HONAVAR, 2008) and the software DNASTAR (Lasergene 14.1).

3.5 INDIRECT ELISA

3.5.1 Chemical synthesis of selected peptides

The peptides selected by immunodetection were chemically synthesized according standard protocol by Fmoc strategy (9-fluorenylmethyloxycarbonyl) using a rink amine resin (Sigma-Aldrich, USA) as insoluble solid support

(Merrifield, 1969) with MultiPep RS automated peptide synthesizer (Intavis Bioanalytical Instruments, Nattermannallee, Germany).

After the last synthesis cycle, the peptides were released from the resin by trifluoracetic acid treatment, filter and precipitate with cold ethyl ether, yielding the peptides. After centrifugation, the ether was discarded and peptides were lyophilized, weighed, dissolved in ultrapure water and stored at -20°C until the next step.

3.5.2 Design of experiments (DoE)

A complete factorial experiment was created to standardize the ELISA's conditions using Minitab© Statistical Software *V. 18 .1* (2017 Minitab, Inc.), with central points included (Table 1). Five factors were considered: antigen concentration, bovine serum albumin (BSA) concentration in blocking solution, serum dilution, conjugated antibody anti-IgG-biotin (Thermo Scientific™) dilution and NeutrAvidin protein conjugate with Horseradish Peroxidase (HRP) (Thermo Scientific™) dilution (Table 2). For each ELISA condition, the response variable was defined as the ratio between the positive and negative sample absorbance readouts.

The experiment was set to maximize the response variable. In cases where the linear fit did not predict the model well, new points were added to fit a correct curvature model.

Table 2. Real and coded values of the independent variables from the full factorial experimental design (2^5) com Ponto central for optimization of the ELISA test (source: author).

Level	Antigen (ng/well)	Blocking solution (% BSA)	Serum (Dilution)	Conjugated antibody (Dilution)	NeutrAvidin (Dilution)
-1	20	3	1:100	1:7500	1:7500
0	60	4	1:200	1:10000	1:10000
1	100	5	1:300	1:12500	1:12500

Was performed in polystyrene, high binding 96 flat bottom wells (MICROLON®, Greiner Bio-One, Germany). Synthetic peptides were diluted in Carbonate coating buffer (0.05 M carbonate, pH 9.6) and incubated overnight at 4 ºC. Wells were washed with washing solution (NaCl 0.9%, tween 20 0.05%). Blocking was performed with 3-5% BSA in PBS (pH 7.4) and incubated for one

hour at 37 ºC. After washing six times, plates were incubated with serum diluted (1:100-1:300) in incubation buffer (0.25% of casein, tween 20 0.05% in PBS pH 7.4) for one hour at 37 ºC. After washing six times, Anti-Human IgG (Fc specific)-Biotin antibody produced in rabbit (Sigma-Aldrich™) was diluted in incubation buffer and added to the plate, following incubation for one hour at 37 ºC. Plates were washed and incubated with neutravidin-HRP (Invitrogen™). The revelation substrate was 3,3′,5,5′-tetramethylbenzidine (TMB) (invitrogen™), and the reaction was stopped with 20 µl of HCl 5 N after 30 minutes. Absorbance was read in spectrophotometer at 450 nm wave-length.

Based on the conditions found (Antigen, blocking solution and serum) in the DoE development ELISA test for anti-covid IgG identification, the development ELISA test for anti-covid IgM identification was carried out, using a secondary antibody anti-Human IgM-HRP (Sigma-Aldrich™)). As this is a variable to be evaluated, the following dilutions of conjugate were considered: 1:15000 to 1:25000 and dilutions of serum 1:100 to 1:300.

3.6 STATISTICS ANALYSES

Results were presented as means ± SD. For the DoE and ANOVA were analyzed using Minitab© Statistical Software *V. 18.1* (2017 Minitab, Inc.). Sensitivity, specificity, and construction of the ROC curve were determined using MedCalc Statistical Software version 18.2.1 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2018).

3.7 ETHICAL ISSUES

The present study was carried out in accordance with the Brazilian national research ethics commission (CEP-CONEP) and the resolution CNS 196/96. Approval was granted by the Ethics Committee from the Federal University of Paraná Process nº 3.954.835.

4. RESULT

4.1 IMMUNODETECTION ASSAY

The spot syntheses generated 316 spots, being each spot a different pentadecapeptide. The reactive epitopes were immunodetected by patients' serum with antibodies for anti-COVID19 (**Fig. 3**.A). The membrane was tested with serum from volunteer health, without antibodies for SARS-CoV-2, in order to verify that these reactions were specific. In this assay was tested the same conditions as one tested for anti-COVID19 (**Fig. 3**.B).

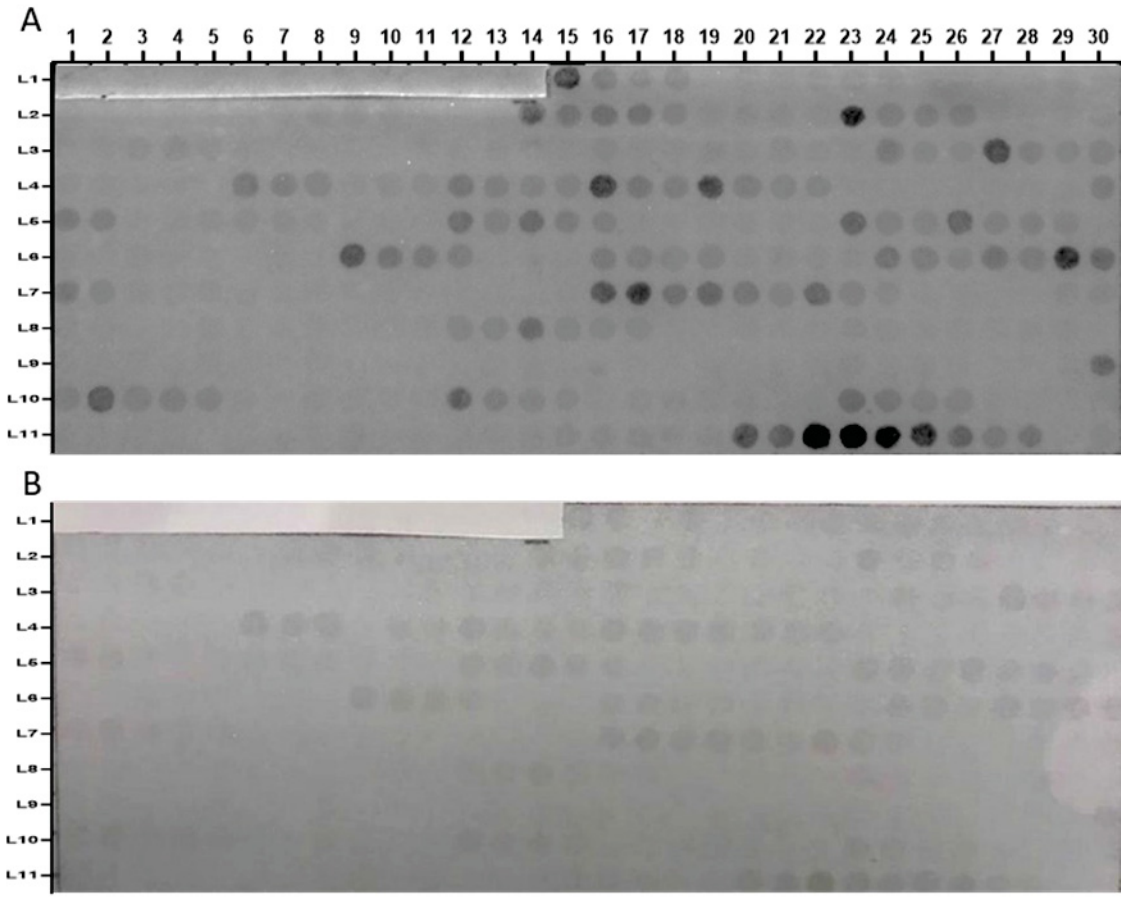

Fig. 3. S Protein of SARS-CoV-2 potential antigenic peptides tested by SPOT-syntheses technique. A. Reactivity of peptides with patients' serum with anti-SARS-CoV-2 IgG antibodies. B. Reactivity of peptides with volunteer health serum (source: author).

When comparing the membrane spots after exposure to positive sera and healthy donor sera (Fig. 4), were found 19.94% (63) positive and 80.06% (253) negative spots were identified. The positive Spots showed a strong signal for peptides corresponding to the regions RBD, monomer-monomer iteration, FS2, and two regions with undetermined functions inside the protein.

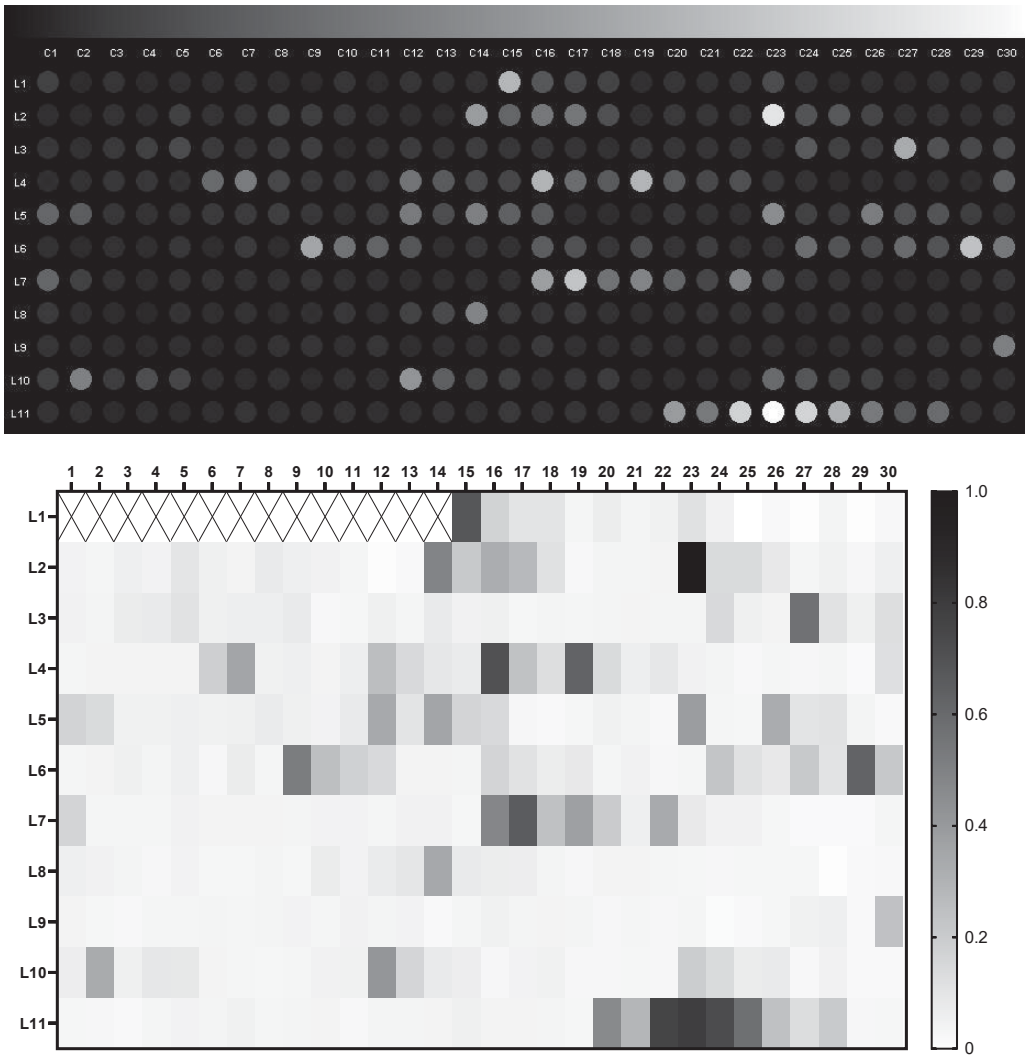

Fig. 4. Comparison of pixel intensity in relation to membrane performance when exposed to serum from patients with COVID19 and serum from healthy volunteers (source: author).

4.2 LINEAR EPITOPE SELECTION IN SILICO

The prediction of linear epitopes of protein S was performed in parallel to the membrane immunodetection of peptides, analyzing the physicochemical (accessibility, hydrophobicity, superficiality) and immunological (epitope, antigenicity) characteristic (respectively). Although the resulting epitope peptides prediction were not totally identical. With the values obtained by the software a Min-max normalization was carry out. The values obtained are separated into two categories, which will be assigned different weights, the first is physicochemical data weighs 33,3% and the second immunological data with 66,7% towards the final value. For each amino acid position were made average of value and multiplied by the weight of the category, to finally add up and get the final value for that position (Fig5 C).

By transforming the variables and combining them were proposed 23 possible B-cell epitope regions with the potential of being core immunogenic which are critical for antibody recognition, considering a cut-off 0.6. these regions corresponding to the region six regions in RBD, eight regions in Monomer-Monomer Interaction (four in S1 and four S2), one in FR1, one in FR2, three glycosylation sites (one in S1 and two S2), and five regions with undetermined functions inside the protein.

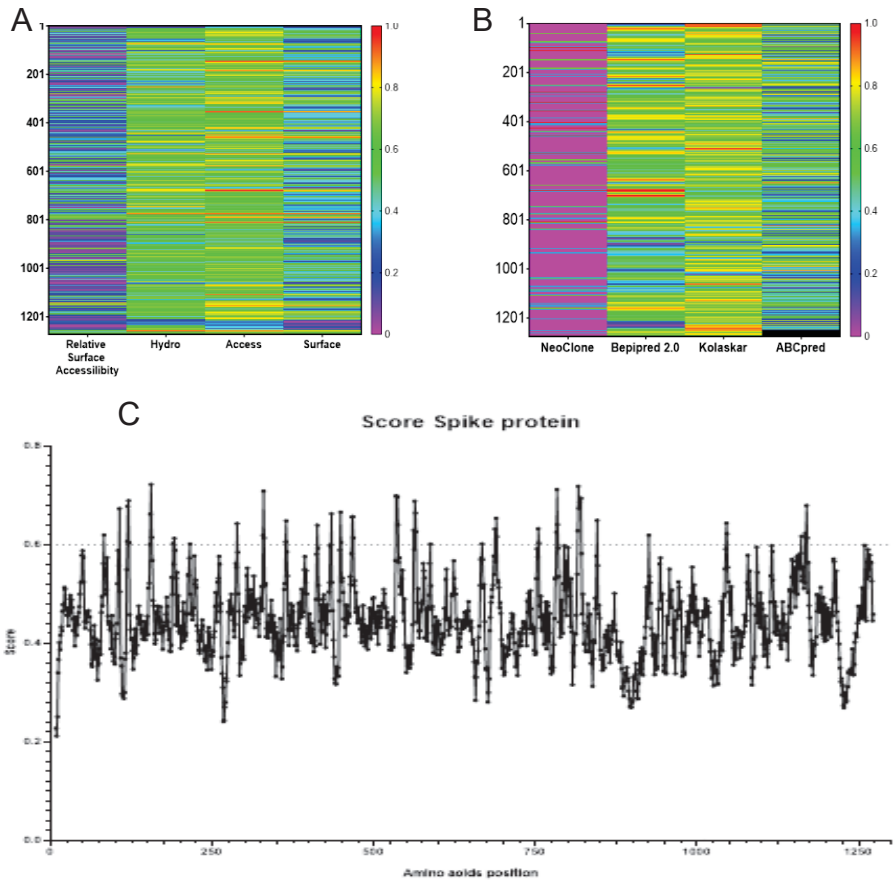

Fig. 5 B Cell Immunodominant prediction of spike protein. A. result of analyzing the physicochemical B. result of analyzing immunological. C. B Cell Immunodominant Regions Based on combination prediction (source: author).

By overlapping the results obtained by bioinformatics and SPOT-synthesis, allowed us to select four regions which are divided into 14 peptides, with ID codes: P.SC2.S.118, P.SC2.S.129, P.SC2.S.130, P.SC2.S.131, P.SC2.S.132, P.SC2.S.136, P.SC2.S.137, P.SC2.S.138, P.SC2.S.202, P.SC2.S.203, P.SC2.S.204, P.SC2.S.205, P.SC2.S.309, P.SC2.S.310, with which we will proceed to perform the standardization of the indirect ELISA test detection of IgG and IgM. When comparing the selected peptides with the main

variants in circulation, it was found that these peptides are located in conserved regions (data not shown).

4.3 ELISA ASSAY

DoE was carried out for the selected peptides, the following pareto plots were obtained (Fig. 6). These results allowed us to identify the ideal conditions for ELISA assay to identification IgG for each of the peptides (Table 3).

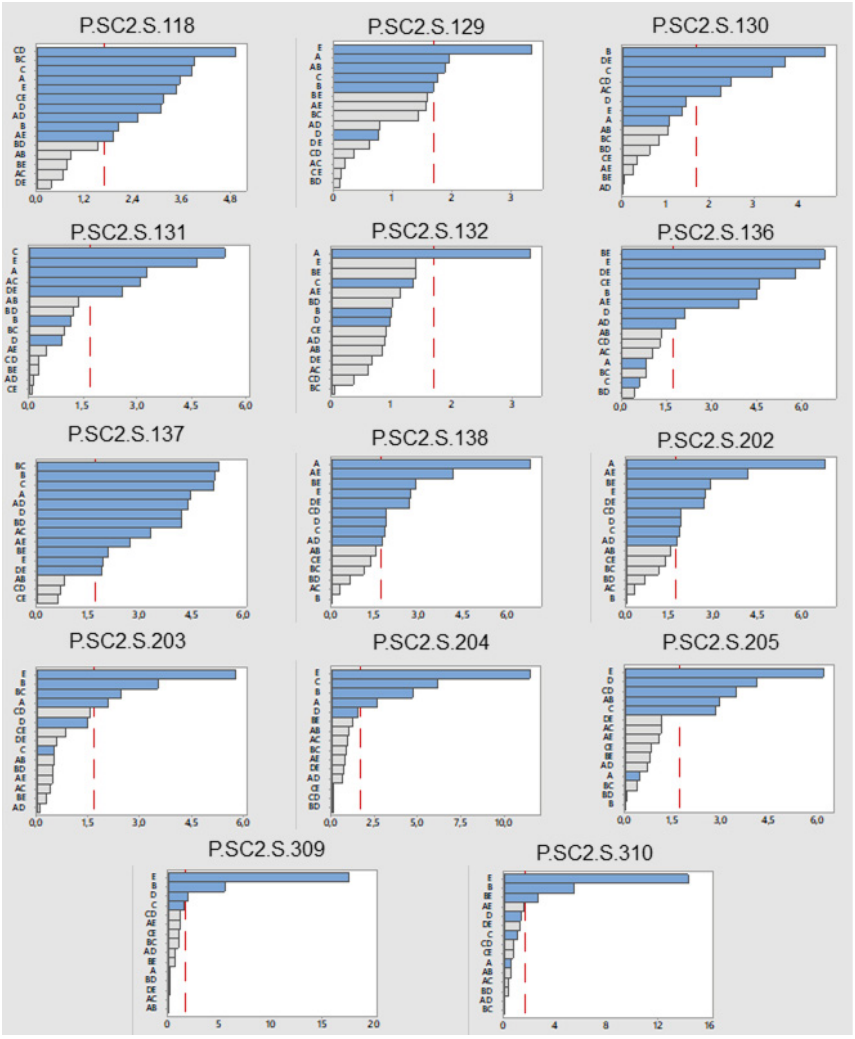

Fig. 6. Pareto chart of the standardized effects from the DoE for the peptides selected: (A) serum, (B) antigen (C) blocking solution, (D) conjugated antibody, and (E) NeutrAvidin (source: author).

The aim is to provide the individual and combined effects of the five independent variables on the response variable for P.SC2.S.203. The ANOVA (presented in ANEXO 1) results showed that the main significant factors were serum, and antigen (symbol S in the last column) are for the dependent variable at 0.05 level of significance. This indicates that different values of the independent

variables serum and antigen result in different performance values for the dependent variable. The interaction antigen*conjugated and Blocking solution*Blocking solution is also significant at 0.05 level of significance while the other combination is not. The R-squared value of 91.09%, states that about 89.64% of the total variability is explained by the model.

Table 3. Optimized indirect ELISA for IgG parameter for peptides calculated by Minitab Statistical Software (source: author).

Peptides	Antigen (Ng/Well)	BSA (%)	Serum (Dilution)	Conjugate antibody (Dilution)	NeutrAvidin (Dilution)	Variation Explained by Model (%)
P.SC2.S.118	20	3	1:100	1:12500	1:7500	78.95
P.SC2.S.129	60	4	1:100	1:10000	1:10000	60.83
P.SC2.S.130	60	4	1:100	1:10000	1:10000	71.82
P.SC2.S.131	100	5	1:100	1:12500	1:7500	86.16
P.SC2.S.132	60	4	1:100	1:10000	1:10000	62.76
P.SC2.S.136	20	3	1:100	1:12500	1:12500	84.46
P.SC2.S.137	20	3	1:100	1:12500	1:7500	91.87
P.SC2.S.138	20	5	1:100	1:7500	1:7500	87.65
P.SC2.S.202	20	5	1:100	1:7500	1:7500	81.18
P.SC2.S.203	20	3	1:100	1:12500	1:7500	89.64
P.SC2.S.204	60	4	1:100	1:10000	1:10000	94.43
P.SC2.S.205	20	5	1:100	1:7500	1:7500	93.96
P.SC2.S.309	20	3	1:100	1:12500	1:7500	93.01
P.SC2.S.310	20	3	1:100	1:7500	1:12500	91.76

The Fig. 7 shows the result of the absorbance of the positive and negative samples tested, for each of the peptides under the conditions determined in DoE. The region that performed best in the ELISA test for IgG identification is the one comprising peptides P.SC2.S.202 to P.SC2.S.205, being peptide P.SC2.S.203, which showed the best ratio 4.41 between positive and negative pool.

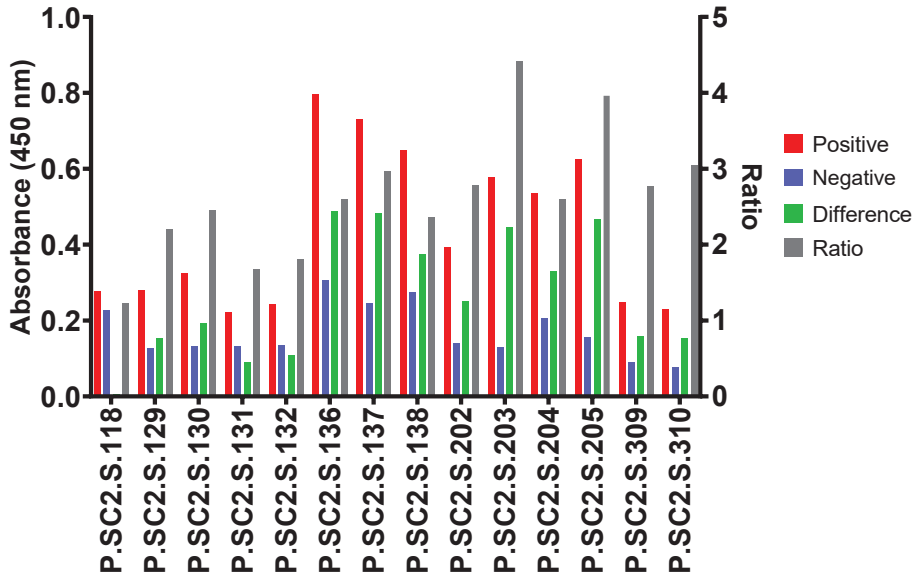

Fig. 7. Absorbance values obtained in the standardization of the indirect ELISA assay using peptides as antigen corresponding to the detection tests for anti-COVID IgG. Positive and negative sera pools were tested at the optimized conditions for each peptide. The difference showed is the absorbance of positive serum minus the absorbance of the negative serum. Rate is the proportion between positive and negative serum absorbance (source: author).

The Fig. 8 shows the result of the absorbance of the positive and negative samples tested, for each of the peptides under the conditions determined (Table 4). The peptides that performed best in the indirect ELISA test for IgM, is P.SC2.S.309, which showed the best ratio 3.16 between positive and negative pool. For this test, the results obtained with the P.SC2.S.203 should also be highlighted, it shows 3.12 ratio. However, this does not exclude the other peptides which show a good ratio but low absorptivity values.

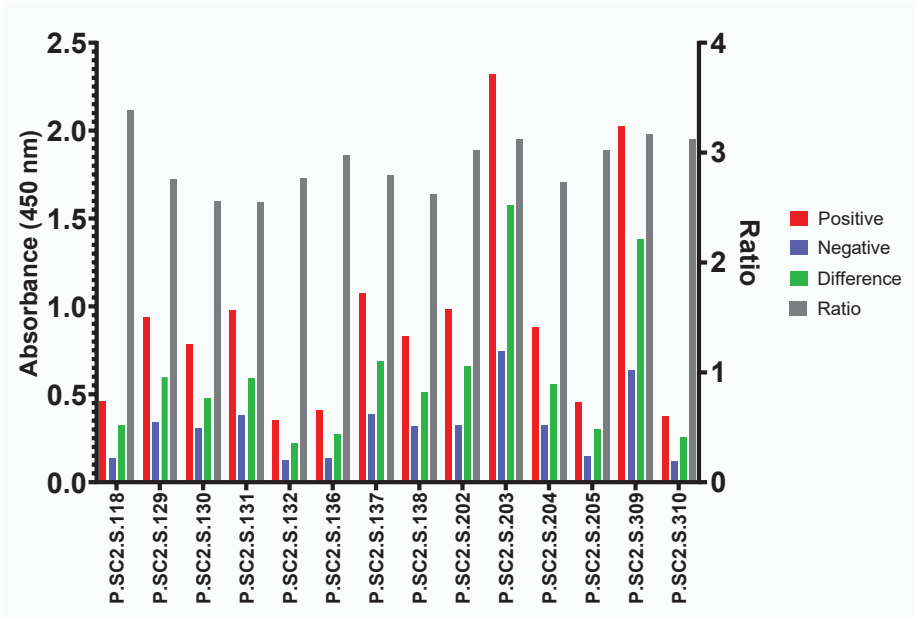

Fig. 8. Absorbance values obtained in the standardization of the indirect ELISA assay using peptides as antigen corresponding to the detection tests for anti-COVID IgM. Positive and negative sera pools were tested at the optimized conditions for each peptide. The difference showed is the absorbance of positive serum minus the absorbance of the negative serum. Rate is the proportion between positive and negative serum absorbance (source: author).

Table 4. Optimized indirect ELISA for IgM parameter for peptides.

Peptides	antigen (ng/well)	BSA (%)	Serum (Dilution)	Conjugate antibody (Dilution)
P.SC2.S.118	20	3	1:200	1:20000
P.SC2.S.129	60	4	1:200	1:15000
P.SC2.S.130	60	4	1:200	1:20000
P.SC2.S.131	100	5	1:200	1:15000
P.SC2.S.132	60	4	1:200	1:20000
P.SC2.S.136	20	3	1:200	1:20000
P.SC2.S.137	20	3	1:200	1:15000
P.SC2.S.138	20	5	1:200	1:20000
P.SC2.S.202	20	5	1:200	1:15000
P.SC2.S.203	20	3	1:300	1:25000
P.SC2.S.204	60	4	1:200	1:15000
P.SC2.S.205	20	5	1:200	1:15000
P.SC2.S.309	20	3	1:300	1:25000
P.SC2.S.310	20	3	1:300	1:25000

Two regions (P.SC2.S.202-205 and P.SC2.S.309-310) were strongly recognized by all two anti-SARS-CoV-2 IgG and IgM after standardization of indirect ELISA test. Two peptides stand out: P.SC2.S.203 for IgG identification and P.SC2.S.309 for IgM. Based on the results obtained, peptides P.SC2.S.203 and P.SC2.S.309 were selected to determine sensitivity and specificity of the indirect ELISA (Fig. 9).

To obtain the cut-off value of the ELISA, 78 random sera were assayed by ELISA. The ROC curve was established by considering results of the patients' diagnoses as true positives and true negatives to pre-pandemic samples, cut-off values were determined using the Youden index, and the results are shown in Fig. 9. The area under the ROC curve (AUC), the highest Youden index with corresponding cut-off value, the sensitivity and specificity of peptides ELISA are shown in Table 5.

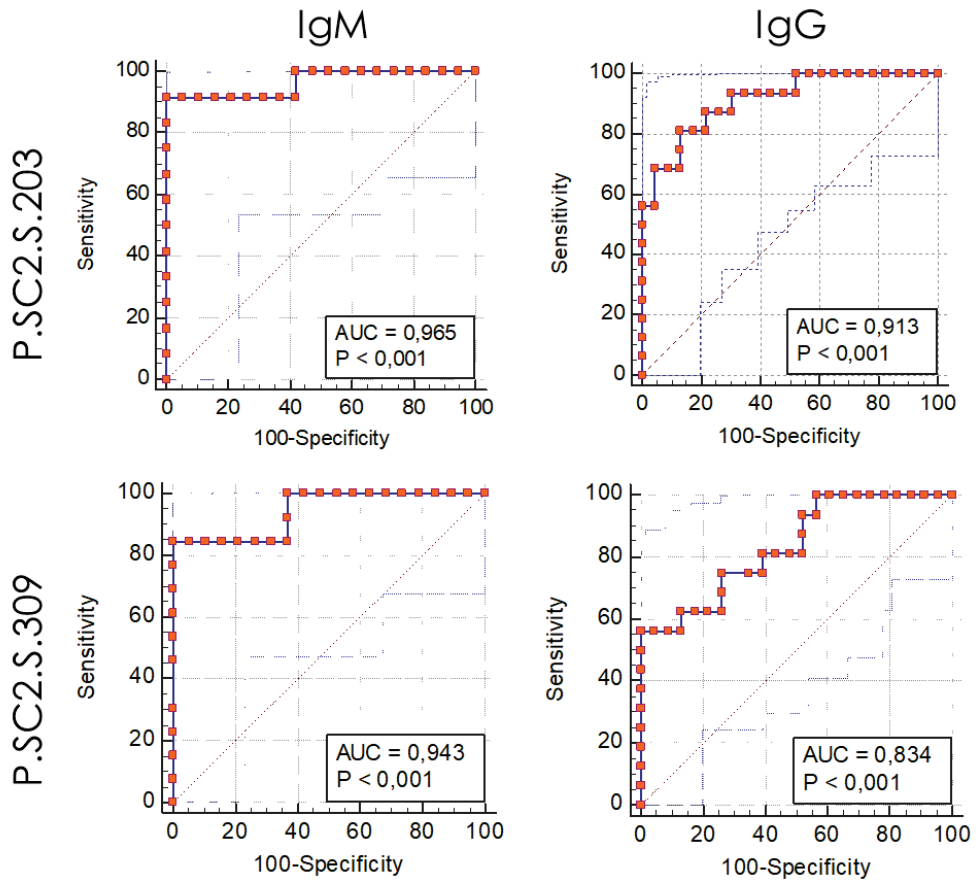

Fig. 9. Receiver operator characteristic (ROC) curve indirect ELISA test using peptides to immunodetect IgM and IgG Anti-SARS-CoV-2 antibodies (source: author).

Table 5 Parameters of the use of peptides for serological diagnosis of COVID-19, sensitivity (Sen.), specificity (Esp.) (source: author).

Peptides	Serological IgM Diagnostic					Serological IgG Diagnostic				
	AUC	Sen. (%)	Esp. (%)	Youden index J	Cut-off	AUC	Sen. (%)	Esp. (%)	Youden index J	Cut-off
P.SC2.S.203	0,965	91,67	100,00	0,916	0,279	0,913	81,25	86,96	0,689	0,373
P.SC2.S.309	0,943	84,62	100,00	0,846	0,332	0,834	56,25	100,00	0,562	0,408

5. DISCUSSION

COVID-19 has now become the most prevalent and widespread respiratory decease in human society. SARS-CoV-2 has been reported in multiple countries with the present of different linages, which increased infectiousness, resistance and the increased likelihood of loss of vaccine effectiveness, resulting in cross-infection with different linage of SARS-CoV-2 in some patients (GONG et al., 2020; LEUNG et al., 2021; SUN et al., 2020).

The development of effective and reliable serological detection methods would play an important role in attending to these needs (GUO et al., 2021). A proposal that we are addressing in this work is the development of serologic diagnostic test based in peptides identification by B-cell epitopes prediction and SPOT-synthesis.

SPOT-synthesis, is a very versatile technique that allows to evaluate the interaction of peptides immobilized on a membrane with a wide variety of molecules (LÓPEZ-PÉREZ et al., 2017). In this work, we focus on the antigen-antibody interaction, which is a vitally important step in the development of immunodiagnostics. This methodology enabled us to screen a large number of peptides simultaneously, at a low cost, and reducing the time required for the development of the assay (HILPERT; WINKLER; HANCOCK, 2007b). This method allowed us to reduce the number of possible epitopes for development of indirect ELISA test by 80%, however, was not conclusive.

An in silico analysis was performed to evaluate some parameters and protein's structure to identified and verified protein regions that manifest epitope-like characteristics. The protein's surface and accessibility of amino acid side chains reflects their potential participation in protein-protein or protein-ligand interactions (VASICEK et al., 2012), the next in evaluation is hydrophobicity is It has taken a great importance for the synthesized peptides, being that these have to be more hydrophilic better and simple are its chemical synthesis and its application as antigens for diagnosis, which leaves us with the following the immunological characteristics are the most important for identifying the regions immunogenic, that are most likely to trigger the humoral immune response. which left us with the following possible immunogenic regions: RBD, Monomer-Monomer Interaction, FR1, FR2, glycosylation site, and some regions with undetermined functions inside the protein.

In RBD region two immunogenic regions (P.SC2.S.118 and P.SC2.S.129 to P.SC2.S.131) were identified and evaluated in an ELISA assay format, although this region is one of the most used for the diagnosis of COVID-19, we found some limitations for the massive use of these peptides, one of them is the presence of substitutions in the protein sequence of the lineage (POKHREL et al., 2021), which could mean the loss of sensitivity and specificity of the tests.

Another region that proved interesting for diagnostic use is the region comprised by peptides P.SC2.S.309 and P.SC2.S.310 because it showed good performance in the identification of anti-COVID IgM antibodies, which is located between the transmembrane domain and the cytoplasmic domain, these domains allow the S protein to anchor to the viral membrane.

The next FR proteolysis, is a region that is related to the binding mechanism of the RBD region with hACE2, since the cleavage of this area is necessary to allow the RBD interaction with hACE2, which makes this region of greater interest. In this region are found peptides P.SC2.S.203 to P.SC2.S.205, which in relation to their performance the region performed very well in the use of indirect ELISA tests showing good sensitivity and specificity.

According to the results from the DoE analysis, the variable with most influence significantly (0.05 level of significance) for indirect ELISA test using P.SC2.S.203 was dilution of antigen, followed by dilution of serum and finally there would be the antigen conjugate interaction. When using the test condition determined by DoE for IgG Anti-SARS-CoV-2 antibodies and IgM Anti-SARS-CoV-2 antibodies using P.SC2.S.203, was the one antigen that presented a higher difference between positive and negative serum; and a rate of 4.41 and 3.12 respectively, achieving one of the most important characteristics of a diagnostic test. Thus, it was demonstrated that P.SC2.S.203 could discriminate between COVID-19 patients and healthy individuals, and with a higher rate than the other peptides described here. For that reason, P.SC2.S.203 was chosen to be further evaluated with a larger number of samples to determine sensitivity and specificity of the methodology, Obtaining 81.28 % and 88.96 % sensitivity and specificity for the IgG detection test and 91.67 % and 100 % for the IgM test, respectively.

There are a wide variety of diagnostic kits for COVID-19 on the market which mainly use protein S as an antigen., having these kits sensitivity and specificity provided by manufactures ranges from 55 to 100% and 85 to 100% respectively (KUBINA; DZIEDZIC, 2020; VEDOVA-COSTA et al., 2021),

To further identify the potential antigen epitope regions of the S protein, in vitro and in silico B cell epitopes by overlapping the sequences generated from the combination of the methodologies used. Based on our results, as peptide P.SC2.S.203 was identified as a potential antigen epitope region. Further

sequence comparison analysis with lineages of SARS-CoV-2 revealed that this region was low variation region, supporting our results of sensitivity and specificity found when testing peptide as an ELISA assay antigen.

6. CONCLUSIONS

In conclusion, the use of in vitro analyzed in conjunction with bioinformatic predictions points to specific regions of S protein of SARS-CoV-2 that have a high likelihood of being used for development a serological test with high probabilities do have high sensitivity a specificity.

Based on the overlapping of the results of the in silico and in vitro tests, it was possible to develop a serological diagnostic method for covid using the P.SC2.S.203 test which showed a sensitivity and specificity of 81.28% and 88.96% sensitivity and specificity for the IgG detection test and 91.67% and 100% for the IgM test, respectively

7. PERSPECTIVES

- The methodology used for identification peptides can be performance for identification new peptides from other proteins of SARS-CoV-2.
- Transfer the use of peptides to other point-of-care platforms such as lateral flow or immunoelectrochemistry**.**
- Test the product in a larger group of individuals how had different linages of SARS-CoV-2.
- Evaluate the peptides identified as possible immunizing agents.

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SUPPLEMENTARY MATERIAL

ANEXO 1: ANOVA result for DoE of P.SC2.S.203

For each main effect, interaction and quadratic effect, the table included the sum of squares (SS), the degree of freedom (DF), the mean square (MS), an F-ratio, calculated using the residual mean square, and the significance level of the P-value.

Source	DF	Adj SS	Adj MS	F-Value	P-Value	
Model	17	17,4759	1,0280	11,42	0,000	
Blocks	1	0,0741	0,0741	0,82	0,376	
Linear	5	3,7089	0,7418	8,24	0,000	
Blocking solution	1	0,1986	0,1986	2,21	0,154	NS
Antigen	1	0,7303	0,7303	8,12	0,010	S
Conjugated	1	0,1043	0,1043	1,16	0,295	NS
NeutrAvidin	1	0,0366	0,0366	0,41	0,531	NS
Serum	1	2,5672	2,5672	28,53	0,000	S
Square	1	12,3841	12,3841	137,62	0,000	
Blocking solution*Blocking solution	1	12,3841	12,3841	137,62	0,000	S
2-Way Interaction	10	0,9538	0,0954	1,06	0,436	NS
Blocking solution*Antigen	1	0,0023	0,0023	0,03	0,874	NS
Blocking solution*Conjugated	1	0,0000	0,0000	0,00	0,986	NS
Blocking solution*NeutrAvidin	1	0,0038	0,0038	0,04	0,838	NS
Blocking solution*Serum	1	0,0021	0,0021	0,02	0,879	NS
Antigen*Conjugated	1	0,4573	0,4573	5,08	0,036	S
Antigen*NeutrAvidin	1	0,0217	0,0217	0,24	0,629	NS
Antigen*Serum	1	0,0002	0,0002	0,00	0,965	NS
Conjugated*NeutrAvidin	1	0,2751	0,2751	3,06	0,097	NS
Conjugated*Serum	1	0,1199	0,1199	1,33	0,263	NS
NeutrAvidin*Serum	1	0,0009	0,0009	0,01	0,922	NS
Error	19	1,7098	0,0900			
Lack-of-Fit	13	1,3244	0,1019	1,59	0,296	
Pure Error	6	0,3854	0,0642			
Total	36	19,1857				

ANEXO 2: Patent 1

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2020 024403 5

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PETICIONAMENTO ELETRÔNICO

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 30/11/2020 às 12:51, Petição 870200150515

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Dados do Pedido

Natureza Patente:	10 - Patente de Invenção (PI)
Título da Invenção ou Modelo de Utilidade (54):	ANTÍGENOS PEPTÍDEOS SINTÉTICOS PARA O DIAGNÓSTICO DO SARS-COV-2
Resumo:	A presente invenção trata da proteção de novos antígenos peptídicos e seu uso em diagnóstico do COVID-19. Os peptídeos compreendem uma sequência de aminoácidos selecionada por bioinformática e spot-synthesis. Os métodos de diagnóstico que podem empregar estes peptídeos são baseados na interação do antígeno com componentes de amostras biológicas e posterior revelação da interação entre estes. Os novos antígenos foram sintetizados quimicamente, avaliado e validado com soro de pacientes COVID-19 positivos e soros colhidos antes do início da pandemia. Os antígenos em questão P.SC2.S.203 (SEQ NO 1), P.SC2.S.130 (SEQ NO 2), P.SC2.S.131 (SEQ NO 3), P.SC2.S. 137 (SEQ NO 4) P.SC2.S.136 (SEQ NO 5), P.SC2.S.138 (SEQ NO 6), P.SC2.S.202 (SEQ NO 7), P.SC2.S.204 (SEQ NO 8) e P.SC2.S.205 (SEQ NO 9), detecta resposta humoral, sendo útil ao diagnóstico do COVID-19 por técnicas imunológicas, como o ensaio imuno-enzimático (ELISA indireto), mas não se limitando a esta. O teste ELISA usando o novo antígeno P.SC2.S.203 (SEQ NO 1) alcança valores de sensibilidade e especificidade para IgM 100.00 e 91,67%, e para IgG 81,25 e 86,96% respectivamente, além dos índices previamente mencionados apresentou índice Kappa com excelente concordância e índice de Youden mostra que o antígeno discrimina perfeitamente os resultados entre doentes e não doentes. A aplicação desta invenção está relacionada com a saúde humana e se situa no campo industrial.
Figura a publicar:	4

ANEXO 3: Patent 2

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Dados do Pedido

Natureza Patente:	10 - Patente de Invenção (PI)
Título da Invenção ou Modelo de Utilidade (54):	PEPTÍDEO SINTÉTICO P.SC2.S.132 PARA KIT ELISA DE AVIDEZ NO DIAGNÓSTICO DE COVID-19
Resumo:	A presente invenção reivindica o peptídeo sintético P.SC2.S.132 para aplicação no sorodiagnóstico por ELISA de avidez pela interação com o anticorpo IgG específicos para SARS-CoV-2. Para tanto, o peptídeo foi engenheirado a partir de uma sequência de aminoácidos da proteína S de SARS-CoV-2, adicionado de outros aminoácidos para conferir características específicas. Em seguida, o peptídeo foi testado frente a soros de pacientes infectados com o vírus, e se mostrou imunorreativo e capaz, pelo teste de ELISA de Avidez, diferenciar pacientes com infecção ativa daqueles potencialmente imunes. Deste modo, o peptídeo demonstrou potencial para ser utilizado como antígeno para diagnóstico sorológico da COVID-19, sendo capaz de diferenciar pessoas com infecção ativa daquelas potencialmente imunes pela presença de anticorpo IgG de alta avidez.
Figura a publicar:	2

ANEXO 4: Patent 3

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29409161921769830

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Dados do Pedido

Natureza Patente:	10 - Patente de Invenção (PI)
Título da Invenção ou Modelo de Utilidade (54):	PEPTÍDEO SINTÉTICO P.SC2.S.129 PARA IMUNODETECÇÃO DE ANTICORPOS IGM, IGA E IGG CONTRA COVID-19
Resumo:	A presente invenção reivindica um novo antígeno o peptídeo sintético, sintetizado por via química ou biológica, P.SC2.S.129 que tem forte interação com anticorpos IgM, IgA e IgG específicos contra COVID-19. Para tanto, o peptídeo foi engenheirado a partir de uma sequência de aminoácidos da proteína S de SARS-CoV-2, adicionado de outros aminoácidos para conferir características específicas. Em seguida, o peptídeo foi testado frente a soros de pacientes infectados com o vírus, e se mostrou imunorreativo. Além disso, o peptídeo foi também testado como antígeno em teste do tipo ELISA, para detecção de Ig Total (IgM, IgA e IgG). Deste modo, o peptídeo demonstrou potencial para ser utilizado como antígeno para diagnóstico sorológico da COVID-19, tanto na fase aguda ou mesmo havendo decorrido algumas semanas da infecção.
Figura a publicar:	2

ANEXO 5: Patent 4

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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29409161921769784

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

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Dados do Pedido

Natureza Patente:	10 - Patente de Invenção (PI)
Título da Invenção ou Modelo de Utilidade (54):	ANTÍGENO PEPTÍDEO SINTÉTICO P.SC2.S.309 APLICADO EM KIT E MÉTODO ELISA PARA DIAGNÓSTICO SOROLÓGICO DE COVID-19
Resumo:	ANTÍGENO PEPTÍDEO SINTÉTICO P.SC2.S.309 APLICADO EM KIT E MÉTODO ELISA PARA DIAGNÓSTICO SOROLÓGICO DE COVID-19. Frente ao panorama da pandemia de COVID-19 há a necessidade de alternativas de diagnóstico. A presente invenção descreve um kit e método diagnóstico sorológico para COVID-19. Esta utiliza como antígeno o peptídeo sintético P.SC2.S.309 em imunoensaios no formato de teste ELISA para identificar imunoglobulina IgM em amostras biológicas de humanos, podendo também ser aplicada como Teste de Avidez. O peptídeo P.SC2.S.309 apresenta sequência artificial altamente similar ao do SARS-CoV-2 e de baixa similaridade com outros organismos próximos. Este kit e seu método de uso apresentam alta sensibilidade (85%) e especificidade (100%) para detecção de IgM. Além de ser um teste de baixo custo e fácil execução, possibilitando um teste diagnóstico eficiente mais acessível.
Figura a publicar:	1

ANEXO 6: Patent 5

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

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Dados do Depositante (71)

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Dados do Pedido

Natureza Patente:	10 - Patente de Invenção (PI)
Título da Invenção ou Modelo de Utilidade (54):	USO DE PEPTÍDEO SINTÉTICO P.SC2.S118 PARA DIAGNÓSTICO DE COVID-19 USANDO PLATAFORMA DE IMUNODIAGNÓSTICO
Resumo:	A presente invenção trata da proteção de novo antígeno peptídico sintético e seu uso em imunodiagnóstico da doença COVID-19. O P.SC2.S.118 compreende uma sequência de aminoácidos detectado pelas metodologias capazes de simular epítopo de SARS-CoV-2. Os métodos de diagnóstico que podem empregar este peptídeo são baseados na interação do antígeno com componentes de amostras biológicas e posterior revelação da interação entre estes. O novo antígeno foi sintetizado quimicamente, avaliado e validado com soro de pacientes positivos para COVID19 e controles negativos. O antígeno em questão P.SC2.S.118 (SEQ ID No. 1) detecta resposta humoral e o estágio da resposta com a identificação das diferentes imunoglobulinas (IgM, IgG e IgTotal), sendo útil ao diagnóstico da COVID-19 por técnicas imunológicas, como o ensaio imuno-enzimático (ELISA indireto), mas não se limitando a esta. O teste ELISA com o uso do novo antígeno P.SC2.S.118 apresentou um índice de avidez de 60% e a relação entre as absorbâncias das amostras positiva e negativa está entre 2 e 4, dependendo do conjugado utilizado. O(s) método(s) e o antígeno usado na presente invenção não apresentam toxicidade, não são invasivos, tornando-se uma opção de diagnóstico mais acessível pelo baixo custo em relação aos métodos moleculares. A aplicação desta invenção está relacionada com a saúde humana e se situa no campo industrial.
Figura a publicar:	2

ANEXO 7: A published article

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Review - 75 years - Special Edition

A Review on COVID-19 Diagnosis Tests Approved for Use in Brazil and the Impact on Pandemic Control

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HIGHLIGHTS

- The main COVID-19 diagnostic methods in Brazil are updated.
- More than 80% of the approved products are from other countries.
- The tests are based on the detection of nucleic-acid (15.8%), antigen (13%) and antibody (71.2%).
- The most of Brazilian federative units show an elevated percentage of cases per samples tested.

Abstract: With the COVID-19 pandemic, many diagnostic tests (molecular or immunological) were rapidly standardised, given the urgency of the situation, many are still in the process of being validated. The main objective of this study was to review the aspects of the diagnostic kits approved in Brazil and their application in the different federative units to gather epidemiological information. In order to achieve these objectives, a survey was carried out on the data available at the regulatory agency (ANVISA) and in the literature. The main countries that have registered products in Brazil are China (51.4%), Brazil (16.6%), South Korea (9.2%), USA (8.8%) and Germany (3.6%). The methodologies of these products are based on the detection of nucleic-acid (15.8%), antigen (13%) and antibody (71.2%). In the immunological tests, it was verified that the sensitivity ranged from 55 to 100% and the specificity from 80 to 100%. The percentage of cases in the samples tested in Brazil is elevated in almost all federative units since eight states showed 40% of positive cases in tested samples, while 18 states displayed between 20 and 40%. In conclusion, this review showed

ANEXO 8: An article under review

New age in vaccinology: the impact of the COVID-19 pandemic on third-generation vaccine development

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Abstract

The emergency caused by the COVID-19 pandemic required fast and effective vaccine development. Thus, third-generation vaccine technologies were pushed forward and, for the first time, obtained approval for human use. This review presents an analysis on the production, efficacy, and safety of different third-generation vaccines for infectious diseases. DNA, mRNA, and viral vector vaccines in phase III and IV clinical trials were explored here, with considerations and comparisons between them. Two databases of clinical trial vaccines were also analyzed, revealing that 7.9% of phase III and IV clinical trial studies are related to third-generation vaccines. Most of these studies started in 2020 (28.6%) and 2021 (63.1%). The target disease is primarily COVID-19 (92.9%), and most trials are concentrated in developed countries. Scientific, economic, and political factors influenced the rapid development of third-generation vaccines, marking the beginning of a new age in vaccinology. The knowledge obtained during this brief period will influence future vaccine development and treatment of infectious diseases.

Keywords: vaccines; mRNA; viral vector; DNA; vaccine production; biotechnology; bioprocess.

1. Introduction