Study of the behavior of SARS-CoV-2 and assessment of health protocols against COVID-19 during the fifth and sixth waves of the pandemic in Camp de Tarragona.

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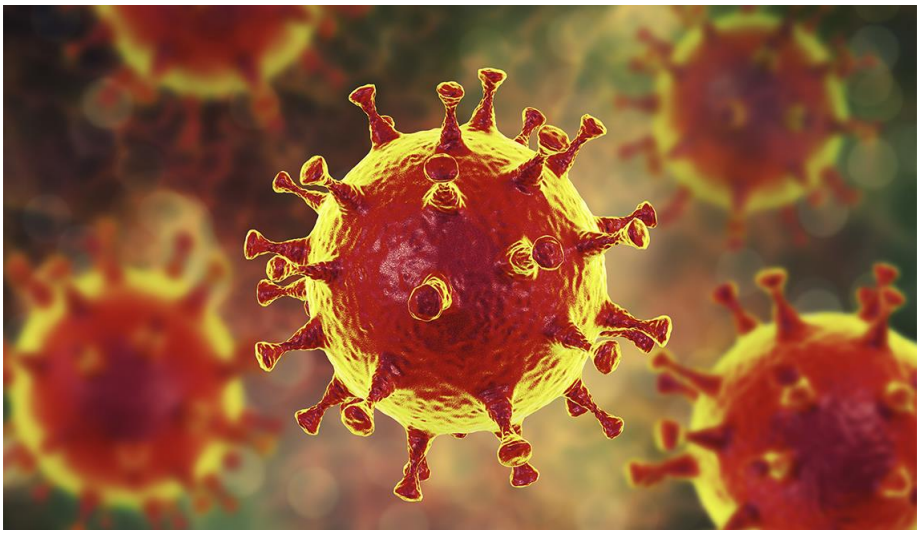
Trabajo de fin de grado de Bioquímica y Biología Molecular



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Junio 2022

Yo, Miguel Boullón Cassau, con DNI 54227519-M, soy conocedor de la guía de prevención del plagio en la URV Prevención, detección y tratamiento del plagio en la docencia: guía para estudiantes (aprobada el julio 2017) y afirmo que este TFG no constituye ninguna de las conductas consideradas como plagio por la URV.

Tarragona, 6 de junio 2022

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Index

Index of tables

Index of figures

Abbreviations

- ACE (angiotensin-converting enzyme)
- AEBM (in spanish, *Asociación Española de Biopatología Médica*)
- AP-1 (activating protein)
- CoV (coronavirus)
- COVID-19 (coronavirus disease 2019)
- Ct (cycle threshold)
- DNA (deoxyribonucleic acid)
- ELISA (enzyme-linked immunosorbent assay)
- Ig (immunoglobulin)
- MERS-CoV (Middle East respiratory syndrome coronavirus)
- MHC (major histocompatibility complex)
- ORF (open reading frame)
- PAMP (pathogen associated molecular patterns)
- PCR (polymerase chain reaction)
- PRR (pattern recognition receptors)
- RBD (receptor binding domain)
- RGQ (Rotor-Gene Q)
- RNA (ribonucleic acid)
- RT-PCR (reverse transcription–PCR)
- SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2)
- ssRNA+ (single-stranded, positive-sense ribonucleic acid)
- TLR (toll-like receptors)
- VOC (variants of concern)
- VOI (variants of interest)
- WHO (World Health Organization)

ELDINE PATOLOGIA

Eldine Patologia *(www.eldinepatologia.com)* is a Pathological Anatomy laboratory based in Tarragona dedicated to histological, cytological and molecular analysis, as well as the study of tissue and cell samples, extracted in surgeries and medical consultations. 90% of the activity is based on the study for the diagnosis of cancerous pathology or infections by microorganisms with annual figures of more than 20,500 biopsies, 23,500 cytologies and 21,000 molecular tests.

In 2020, they began to offer diagnostic and antibody tests of SARS-CoV-2. This work has been carried out based on the results obtained in the internship under the supervision of Dr. Francisco Algaba Chueca.

ABSTRACT

During December 2019, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) appeared. To fight against this new coronavirus, new health protocols were created to adapt to the new situation. However, since the virus started mutating and new variants appeared, new protocols had to be put in place. The **main objective** of this work is to study the behavior of SARS-CoV-2 throughout the fifth and sixth waves of pandemic in a cohort of 81 patients from Eldine Patología (13 from the fifth and 68 from the sixth wave) that had taken two or more RT-PCR tests in a maximum period of 15 days, of which at least the first one was positive, as well as to compare those results with the indications of the health protocols that the *Generalitat de Catalunya* published at those times regarding quarantine time. The results indicate that the recommended quarantine for the fifth wave is enough (10 days) since all patients were negative at that time. However, some patients from the sixth wave (22,2%) were still positive after the recommended quarantine for that wave (7 days). After 10 days, the rate of positives from the sixth wave decreased to 6%. Moreover, it is demonstrated that there is more than one variant in the sixth wave. Overall, this work indicates that the quarantine time for the fifth wave was more adequate regarding the virus clearance.

Durante diciembre de 2019 apareció el Síndrome Respiratorio Agudo Severo Coronavirus 2 (SARS-CoV-2). Para luchar contra este nuevo coronavirus se crearon nuevos protocolos sanitarios para adaptarse a la nueva situación. Sin embargo, dado que el virus comenzó a mutar y aparecieron nuevas variantes, se tuvieron que implementar nuevos protocolos. El **objetivo principal** de este trabajo es estudiar el comportamiento del SARS-CoV-2 a lo largo de la quinta y sexta ola de pandemia en una cohorte de 81 pacientes de Eldine Patología (13 de la quinta y 68 de la sexta ola) que habían tomado dos o más pruebas RT-PCR en un plazo máximo de 15 días, de las cuales al menos la primera era positiva, así como comparar esos resultados con las indicaciones de los protocolos sanitarios que la *Generalitat de Catalunya* había publicado en aquellos momentos en relación con el tiempo de cuarentena. Los resultados indican que la cuarentena recomendada para la quinta ola es suficiente (10 días) ya que todos los pacientes dieron negativo en ese momento. Sin embargo, algunos pacientes de la sexta ola (22,2%) seguían dando positivo después de la cuarentena recomendada para esa ola (7 días). Después de 10 días, la tasa de positivos de la sexta ola disminuyó al 6%. Además, se demuestra que hay más de una variante en la sexta ola. En conclusión, este trabajo indica que el tiempo de cuarentena para la quinta ola fue más adecuado en cuanto a la eliminación del virus.

Durant el desembre de 2019, va aparèixer el Coronavirus 2 de la síndrome respiratòria aguda severa (SARS-CoV-2). Per lluitar contra aquest nou coronavirus, es van crear nous protocols sanitaris per adaptar-se a la nova situació. Tanmateix, com el virus va començar a mutar i van aparèixer noves variants, s'han hagut de posar en marxa nous protocols. **L'objectiu principal** d'aquest treball és estudiar el comportament del SARS-CoV-2 al llarg de la cinquena i sisena onada de pandèmia en una cohort de 81 pacients d'Eldine Patología (13 de la cinquena i 68 de la sisena onada) que havien pres dos o més proves de RT-PCR en un període màxim de 15 dies, de les quals almenys la primera va ser positiva, així com comparar aquests resultats amb les indicacions dels protocols sanitaris que la *Generalitat de Catalunya* va publicar en aquells moments respecte al temps de quarantena. Els resultats indiquen que la quarantena recomanada per a la cinquena onada és suficient (10 dies) ja que tots els pacients eren negatius en aquell moment. Tanmateix, alguns pacients de la sisena onada (22,2%) encara eren positius després de la quarantena recomanada per a aquesta onada (7 dies). Després de 10 dies, la taxa de positius de la sisena onada va disminuir fins al 6%. A més, està demostrat que hi ha més d'una variant a la sisena onada. En general, aquest treball indica que el temps de quarantena per a la cinquena onada va ser més adequat pel que fa a l'eliminació del virus.

1.INTRODUCTION

At the end of December 2019 in Wuhan, China, several reports of patients that presented pneumonia of unknown etiology appeared. Chinese scientists identified the agent as a new coronavirus (CoV) and its genomic sequence was made public after sequencing clinical samples from a cohort of patients with pneumonia in Wuhan.[1] This disease spread rapidly, not only through China, but through the whole world. What started as a local new disease, soon became a global pandemic. The World Health Organization (WHO) announced the official name of the new disease as "coronavirus disease 2019" (COVID-19) and *The International Committee of Taxonomy of Viruses* named it SARS-CoV-2.[2]

CoVs are enveloped, single positive-strand RNA viruses belonging to the large subfamily Coronaviridae, which can infect mammals and several other animals. Seven CoVs are known to cause human disease and can be divided into low and high pathogenic CoVs. Including SARS-CoV-2, three novel zoonotic CoVs have emerged during the last 18 years, which are considered lethal human diseases. The SARS coronavirus (SARS-CoV), now named SARS-CoV-1, was discovered in November 2002, and the Middle East respiratory syndrome coronavirus (MERS-CoV) in June 2012. Both caused local outbreaks and were contained before causing a pandemic.[2]

COVID-19 shows a complex profile with many different clinical presentations. Similar to many other viral infections, the characteristics of currently infected patients and their clinical outcomes may represent the tip of the iceberg (Figure 1). Patients may be asymptomatic or experience mild to severe symptoms such as pneumonia.[2]

To date (5th June 2022), there have been 222,394,439 total cases with 2,018,509 deaths throughout the world, which has turned this pandemic into a worldwide sanitary and economical crisis.[3] The high number of asymptomatic cases makes the eradication of this virus even more difficult. However, thanks to the vaccines recently developed, the situation has vastly improved since the beginning of the pandemic.

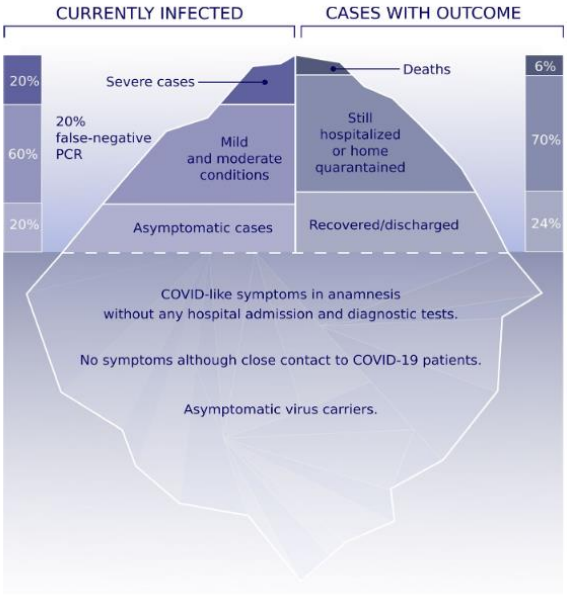

Figure 1: The iceberg of Covid-19 pandemic.[2]

1.1 Structure of Sars-CoV-2

The Sars-CoV-2 belongs to the largest family of the RNA viruses with a genome ranging from 27 to 32 kilobases in size (approximately 125 nm).[4] The virus possesses a single-stranded, positive-sense ribonucleic acid (ssRNA+) as its genome[5], which can be divided into three zones (Figure 2). The first two (closer to the 5' end) encode for the viral replicase gene. This gene is formed by two ORFs (ORF1a and ORF1b). The third part of the genome encodes the 4 main structural proteins (protein S, M, E and N) and other accessory proteins, including protein 3a or 7a.[2]

The virus is a crown-like particle (this is where the name coronavirus comes from) whose diameter varies between 60 and 140nm. The structure consists of a nucleocapsid (protecting the genetic material) and an external sheath made up of a lipid bilayer[2] (Figure 3).

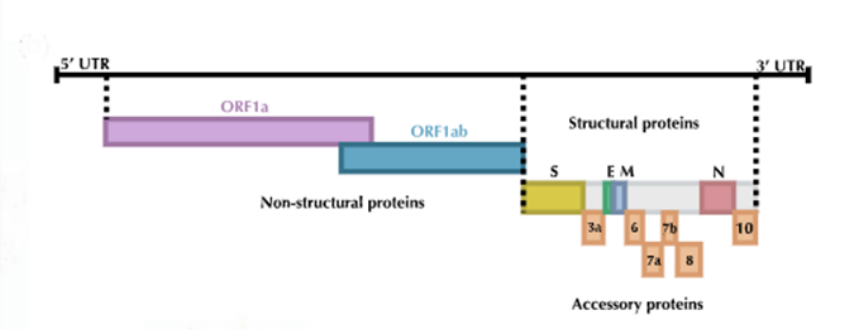

Figure 2: Genetic structure of Sars-CoV-2[6]

The functions of these structural proteins are the following [2,4–6]:

- **Spike protein (S):** this protein plays the most important role in viral attachment, fusion, and entry. Its 30 kb genome RNA is large enough to produce a positive sense to be read directly by ribosomes in the cell. This protein helps in the attachment of the virus to the host cells.
- **Nucleocapsid protein (N):** it ties the viral genome to the replicase-transcriptase complex and packs the encapsulated genome into viral particles to protect it from degradation.
- **Envelope protein (E)**: it is a transmembrane protein which facilitates assembly and release of the virus.
- **Membrane protein(M):** promotes membrane curvature and it binds to the nucleocapsid.
- **Hemagglutinin-esterase dimer protein (HE):** together with the spike protein, it forms the phospholipid bilayer and it is thought to enhance S protein-mediated cell entry and virus spread through mucosa.

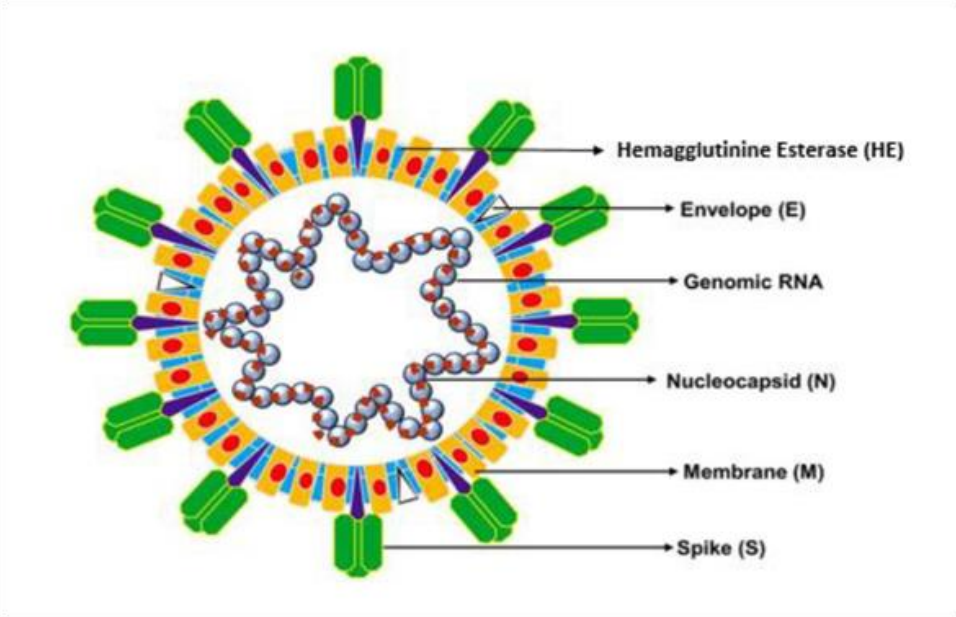

Figure 3: Structure of Sars-CoV-2[7]

1.2 Infection mechanism

The main way of transmission of the virus is through the inhalation of respiratory droplets from one infected person to another, infecting the epithelial cells in the lower respiratory system. For the infection to start, it is necessary for the virus to find a receptor on the host cell membrane (Figure 4). The S protein of the Sars-CoV-2 can bind to the angiotensin-converting enzyme 2 (ACE2) in the host cells.[2] ACE2 is a type I transmembrane protein, expressed in several organs, with the highest levels in the cardiovascular system, intestine, kidneys, brain, testicles and lungs.[8] The S protein has two subunits: S1 and S2. The former one binds to the receptor ACE2 through the binding domain of the receptor (RBD), whereas the latter determines the fusion of the virus's membrane with that of the host's. For the virus to be able to enter the host cell completely, the S protein must be cleaved by the host protease enzyme, TMPRSS2. This cleavage ocurres in two different places of the S2 subunit, which allows the entry of the virus through endocytosis.[2]

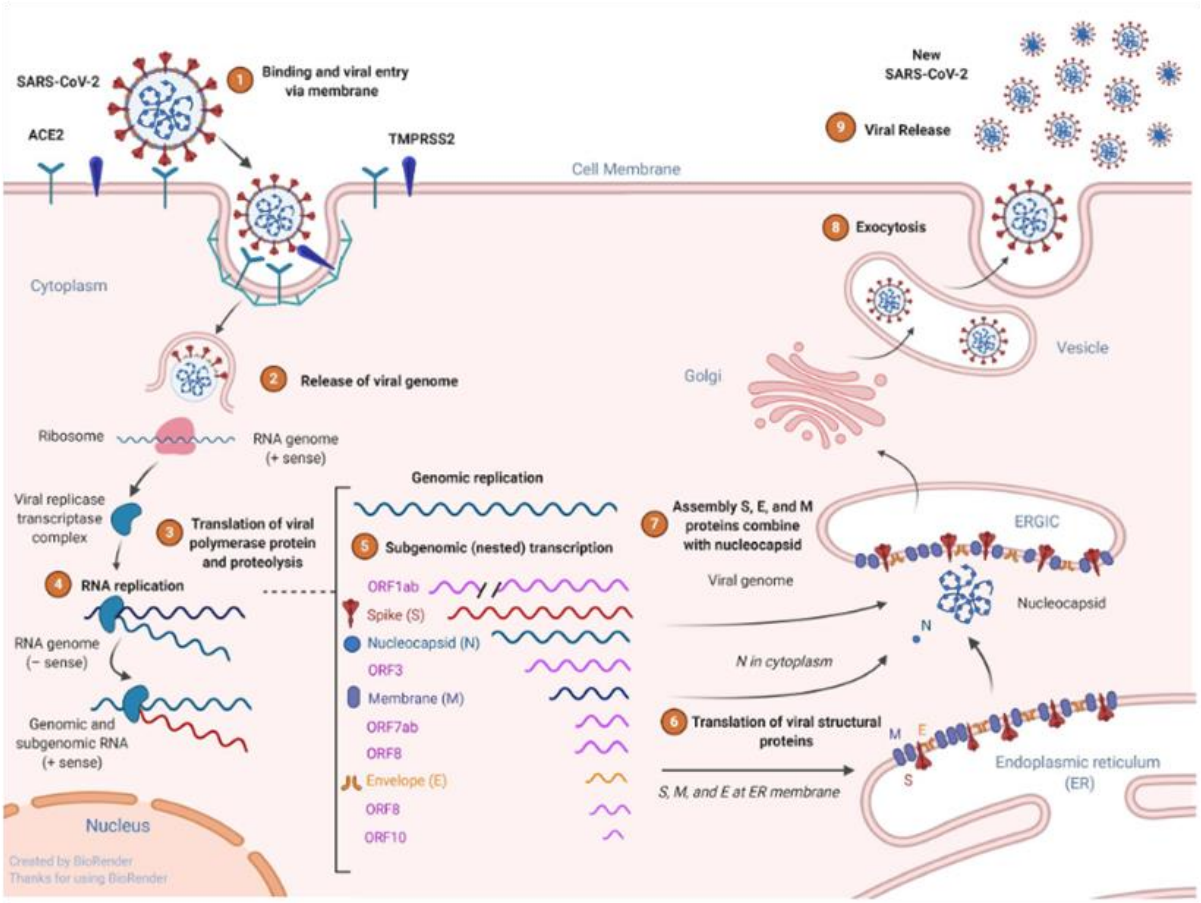

Figure 4: Representation of the infection mechanism from the binding and viral entry, through genomic replication, to viral release.[1]

Once the virus is inside the host cell it releases the ssRNA+ strand which is translated into the viral replicase transcriptase complex. With this complex, the +RNA strand is used as a mold for a -RNA strand. Using the -RNA strand as a new mold, the virus can start replicating its RNA and, once it has been replicated, the new viral proteins are translated in the endoplasmic reticulum where they are sent to the Golgi apparatus. Here the membrane proteins assemble with the nucleocapsid to create a new virus particle which then exits the host cell through exocytosis.[1]

1.3 Immune system response

The human body displays one of two types of immune response when affected by SARS-CoV-2, either the cellular or the humoral response.

1.3.1 Cellular response

To start an antiviral response, the innate immune system detects the infection through pattern recognition receptors (PRRs). The main known PRRs are the Toll-like receptors (TLR), transmembrane proteins with an external domain that binds with pathogen associated molecular patterns (PAMPs). When TLRs are activated, they trigger signaling pathways that activate factors like NF-kB and AP-1, which stimulate the production of inflammatory proteins such as cytokines (IL-1, IL-6 and IL-12). Other factors that are activated in this process are IRF3 and IRF7 that suppress replication of the virus at early stages.[2]

Once the virus is inside the tissue cells, viral peptides are presented through class I major histocompatibility complex (MHC) proteins to CD8+ cytotoxic T cells. CD8+ T cells become activated and start to divide and develop virus-specific memory T cells to prevent future infections. CD8+ cytotoxic T cells lyse the virus-infected tissue cells. For a short time, the whole virus and viral particles are recognized by professional antigen-presenting cells, which are mainly dendritic cells and macrophages, who then present viral peptides to CD4+ T cells through MHC-Class-II molecules. B cells can directly recognize the viruses and get activated by them.[1]

However, in severe cases of COVID-19, there is an overreaction of the immune system, leading to elevated numbers of B cells, T cells, NK cells, macrophages and dendritic cells, leading to an exaggerated cytokine release. The exaggerated cytokine release in response to viral infection, a condition known as cytokine release syndrome or cytokine storm, is emerging as one of the mechanisms leading to the respiratory distress síndrome and multiple organ failure in COVID-19.[9,10]

1.3.2 Humoral response

The humoral response provides adaptive immunity against a specific virus through the creation of specific antibodies or immunoglobulins (Igs) which are produced by B cells that have been activated by the virus directly or through interactions with CD4+ T cells. There are 5 isotypes of antibodies (IgM, IgA, IgG, IgD and IgE) but the first three are the most significant.[11]

- IgM: They appear during the acute phase of the disease (around 5-6 days after viral infection), covering the pathogen's surface forming immune complexes. After the acute phase, they disappear.
- IgA: They neutralize the attachment of the viruses to the mucosal surfaces.
- IgG: They appear after IgM and are responsible for the lifelong protection against this virus.

As shown in Figure 5, IgM appear before IgG but also disappear rapidly, whereas IgG take longer to appear but stay in the body.

Antibodies against SARS-CoV-2 have a neutralizing action over the virus, primarily targeting the S and N proteins, and preventing its binding and invasion of the host cells.[12]

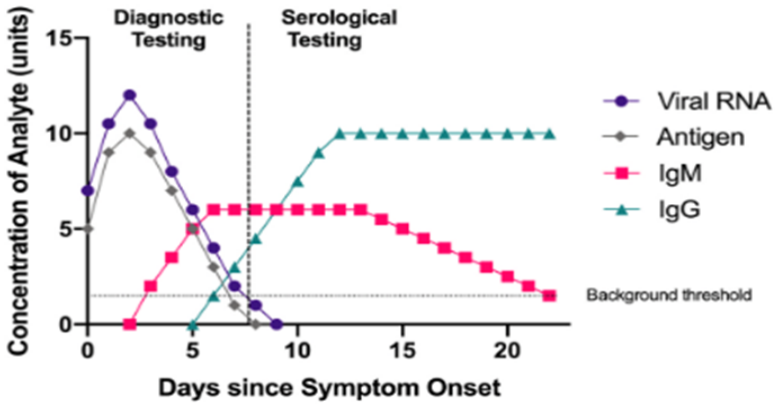

Figure 5: Specific antibody response to SARS-CoV-2[13]

1.4 Variants and waves

Genomes of viruses such as SARS-CoV-2 can alter their sequence during replication in host cells, referred to as mutations. A population of coronaviruses that inherit the same distinctive mutations is called a variant. Several mutations and variants of SARS-CoV-2 have arisen throughout the world over the course of the pandemic. SARS-CoV-2 mutates slower than most RNA viruses due to the proofreading function it has, allowing it to have a higher accuracy in virus replication with fewer mutations. The first variants appeared in March 2020 with a single mutation in the S glycoprotein (D614G). Variants can be classified into two groups: variants of concern (VOC) and variants of interest (VOI). VOC are those variants that increase in transmissibility and in fatality and significantly decrease the effectiveness of

vaccines whereas a VOI is a variant with a genetic capability that affects characteristics of the virus such as disease severity, immune escape, transmissibility and diagnostic escape. Some new SARS-CoV-2 variants are shown in Table 1.[14] However, the Delta and Omicron variants are the ones focused on since this study was performed using data from the fifth and sixth waves in Spain, in which these two variants predominated.

Table 1. Examples of SARS-CoV-2 VOC[14]

Name	Country of Origin	Mutations in Spike Protein	Effect on Monoclonal Antibody Treatment Regimens and Neutralization of Convalescent Sera
B.1.1.7 (Alpha) †	United Kingdom	N501Y *, A570D, D614G, P681H *, T716I, S982A, Δ69/70 *, Δ144 *	1. Retains susceptibility to EUA monoclonal antibody treatments [204] 2. Modest reductions in the neutralizing activity of plasma from convalescent patients (2.7–3.8-fold) [201,202]
B.1.351 (Beta) †	South Africa	D80A, D215G, Δ241/242/243, K417N *, E484K *, N501Y *, D614G, A701V	1. Activity of LY-CoV555 (Bamlanivimab), and REGN10933 (Casirivimab) completely abolished [204] 2. Significant decrease in susceptibility to the combination of bamlanivimab and etesevimab monoclonal antibody treatment [204] 3. The combination of casirivimab and imdevimab appears to retain activity [204] 4. Markedly more resistant to neutralization by convalescent plasma (9.4-fold) [201]
P.1 (Gamma) †	Japan/Brazil	L18F, T20N, P26S, D138Y, R190S, K417T *, E484K *, N501Y *, D614G, H655Y, T1027I	1. Marked reduction in susceptibility to bamlanivimab and bamlanivimab plus etesevimab in vitro [204] 2. Reduction in casirivimab activity, although the combination of casirivimab and imdevimab appears to retain activity [204] 3. Reduced neutralization by convalescent and post-vaccination sera [217]. 4. Neutralizing activity was lower by factor of: a. BNT162b2: 6.7 b. mRNA-1273: 4.5
B.1.617.2 (Delta) †	India	L452R *, E484Q *, D614GD111D, G142D, P614R, P681R *	1. Abolished neutralizing activity of bamlanivimab [234] 2. Partially evaded neutralization by the antibodies induced through natural infection [234]

- **Delta variant (B.1.617.2):** The Delta variant was discovered for the first time in India in late 2020 and it has been said to be 40% to 60% more infectious than the alpha variant. It has 23 mutations compared to the first identified COVID-19 strain out of which twelve are in the spike protein (some of them are shown in Figure 6).

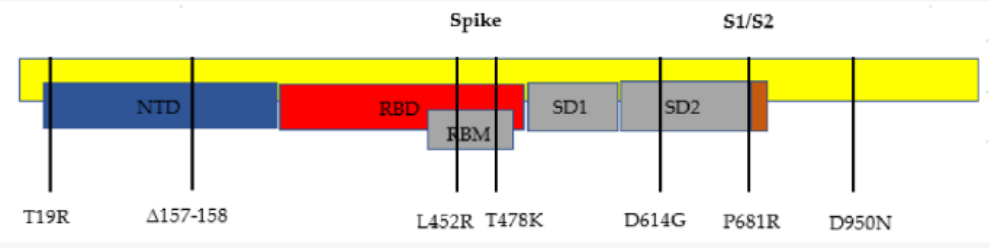

Figure 6: Some mutations in the spike protein in the Delta variant[15]

These mutations in the spike protein allow this variant to be one of the most transmissible variants yet.

One of the most notable mutations is the L452R spike protein mutation. The L452R mutation substitutes an arginine for a leucine at position 452. This allows for the spike protein to attach to the ACE2 receptor with a higher affinity. This may help evade vaccine-stimulated antibodies to bind to the spike protein, because the ACE2 receptor is bound with the spike protein with a higher affinity. It has also been shown that the L452R mutation can allow the Delta variant to evade being attacked by CD8 T cells, which are the cells that eradicate the virus.[15] The Delta variant was the main SARS-CoV-2 variant in which was considered the fifth wave in Spain, corresponding to the time period from June 16th to August 14th.

- **Omicron variant (B.1.1.529):** This variant was first reported in November 2021. Seeing how the Omicron variant is spreading, it is probably more infectious than the Delta and Beta variants. It is also noteworthy that a recent retrospective study based on the population-wide epidemiological data in South Africa indicates an increased risk of SARS-CoV-2 reinfection associated with Omicron. Figure 7 shows the speed of spreading of some of these variants.[16]

Overall, more than 60 substitutions/deletions/insertions have been identified in the Omicron variant, making Omicron a variant possessing the largest number of mutation sites of all SARS-CoV-2 variants characterized so far. As it has already been said, the RBD is the virus entity that recognizes the ACE2 receptor to mediate virus entry. While the Delta variant only possesses the L452R and T478K mutations in the

RBD, 15 mutations have been accumulated in the RBD of the Omicron variant[16] (Figure 8). The Omicron variant was the main SARS-CoV-2 variant in which was considered the sixth wave in Spain, corresponding to the period from December 1th to January 31st.

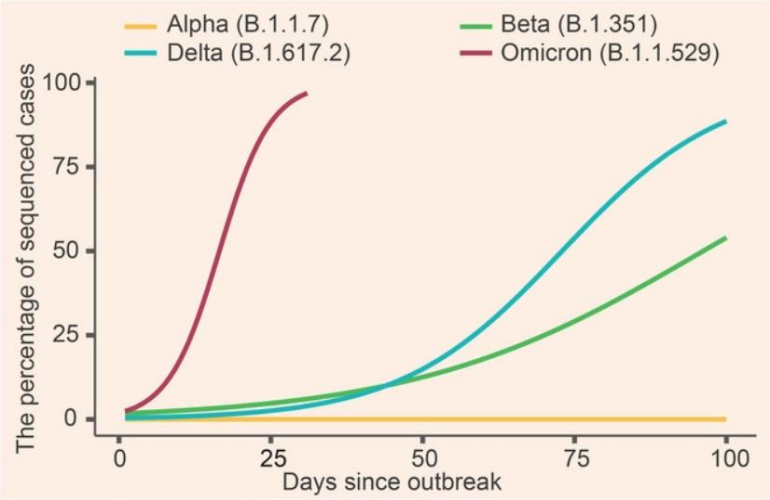

Figure 7: Speed of spreading of some of the mentioned variants[16]

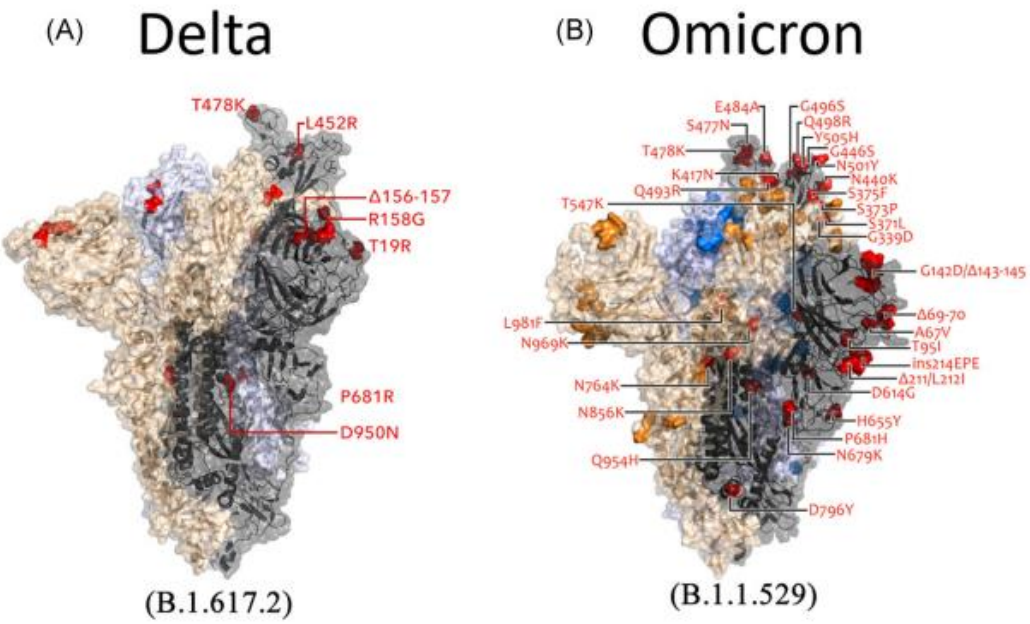

Figure 8: Differences between the mutations in the spike protein between the Delta (A) and Omicron (B) variants.[17]

1.5 Health protocols for the fifth and sixth waves

The government of the *Generalitat of Catalunya* published official health protocols for the fifth and sixth waves, characterized by the main presence of the Delta and Omicron variants, respectively.[18] However, it is important to mention that during the sixth wave both Delta and Omicron variants were present, although the latter was predominant. The mentioned documents are extensive and indicate how to act in the event of a positive diagnostic result for the different population groups (i.e. health professionals, teachers, students and general population). The aim of this strategy for identifying cases and contacts is to increase the ability to identify and trace transmission chains, adapt the response of the health and social system to each epidemic moment and accompany and give comprehensive support to the isolation of cases and contacts.

Both protocols have a series of differences out of which some can be highlighted:

1. Both state that a positive result in any individual makes it a confirmed case. However, the protocol from the sixth wave specifies that the diagnostic test on close contacts should ideally be between the 4th and 6th day from the date of last contact with the confirmed case whereas the protocol of the fifth wave doesn't specify any duration.

2. Since more and more people are fully vaccinated in the sixth wave, the protocol during that period considers that people who have received a complete vaccination and that are considered close contact of a confirmed case will be exempt from quarantine only being able to do essential activities and constantly using a mask.

3. Healthcare professionals in the fifth wave who were properly vaccinated and had a close contact in the workplace, were exempt from quarantine and could continue working but in the sixth wave, if they had been in close contact, they had to follow the general recommendations for quarantine and testing for the general population.

4. Finally, the most significant difference between the two health protocolos is the fact that in the fifth wave, the recommended quarantine time was 10 days whereas in the sixth wave, it was reduced to 7.

1.6 Diagnostic test

There are numerous techniques to detect the virus. Each one of them has a different specificity, detecting different target molecules from SARS-CoV-2 or antibodies from the patient. The former ones, are virus detection techniques while the latter ones are serological tests.[19]

This study will focus on the main virus detection techniques which are the nucleic acid-based test and the antigen test.

1.6.1 Nucleic acid-based tests

The molecular techniques use the genetic material of the virus and are based on the principle of the specificity of base pairing with homologous strands. From the genetic sequencing of SARS-CoV-2, different genetic material-based detections have been proposed, including methods based on polymerase chain reaction (PCR). The viral nucleic acid-based test using quantitative reverse transcription–PCR (RT-qPCR) is the first line screening method of choice for SARS-CoV-2 detection and quantification.[19]

It has three basic steps (Figure 9):

1. **Preparation**: After obtaining a respiratory tract sample from the patient, the viral RNA is extracted so it can be reverse transcribed to cDNA, using the reverse transcriptase enzyme. To do so, the capsid must be broken using a lysis buffer.

2. **Amplification**: Once the cDNA is obtained, it must be amplified so as to have enough signal to be able to detect. To do so, the sample is inserted into a thermo cycler where, following a series of changes in temperature and using specific primers and the Taq polymerase enzyme, millions of copies are made.

3. **Results**: The primers are binded to fluorophores which, once the primer binds to the viral RNA, are activated, emitting fluorescence that is detected by probes. The less cycles from the thermo cycler it takes to detect this fluorescence, the more viral load the patient has.

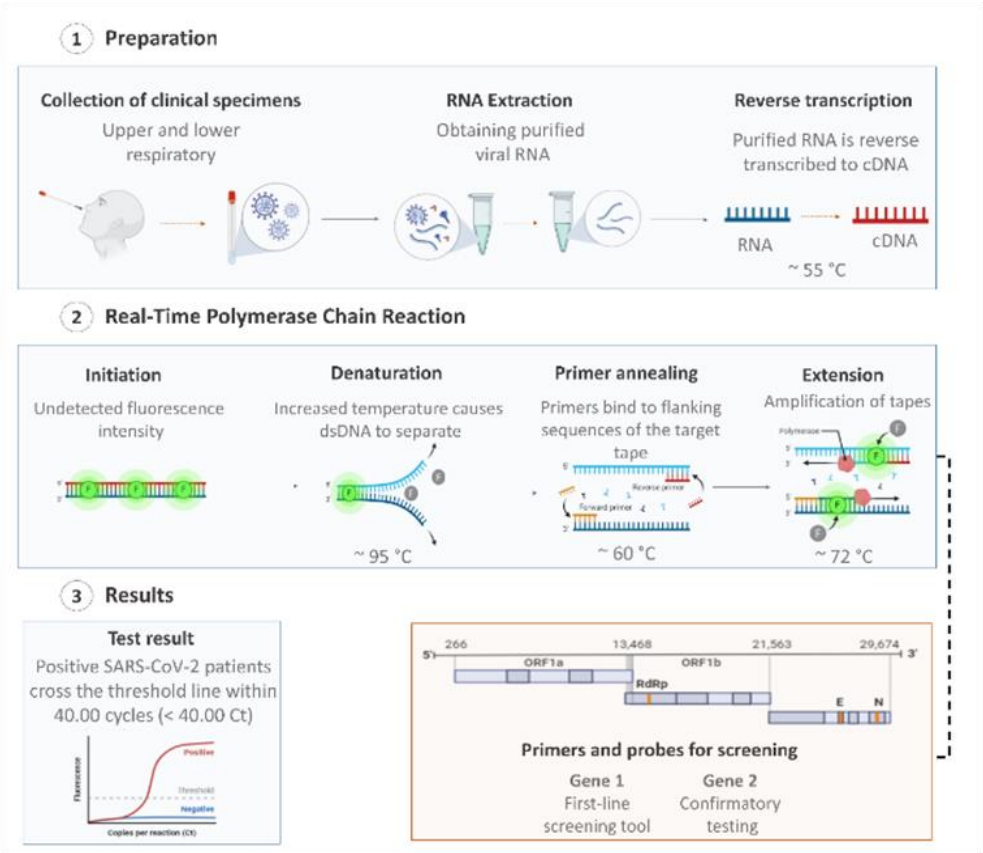

Figure 9: Overview of COVID-19 diagnosis using the reverse transcription–polymerase chain reaction (RT-qPCR) technique with respiratory tract specimens.[19]

1.6.2 Antigen tests

In general, antigen tests are immunoassays that are capable of detecting the presence of a specific viral antigen, which implies a current viral infection. The antigenic assays use different formats, such as ELISA (Enzyme-Linked ImmunoSorbent Assay) or chemiluminescent immunoassay, both for the detection of SARS-CoV antigens.[19] Another format would be the lateral flow immunochromatography, which is the format used in the antigen tests commercially available to the general population. To detect the viral antigen, specific antibodies are used. As can be seen in Figure 10, there are many different ELISA techniques to detect the virus.

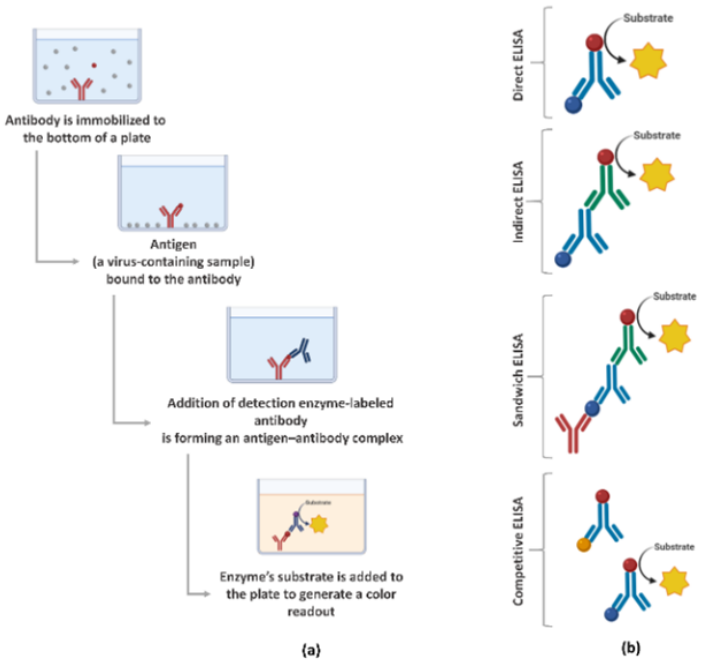

Figure 10: Antigenic test using an ELISA technique.[19]

The result of these tests are based on the detection of the N protein of the virus as well as the detection of a human epithelial antigen that will act as a control, indicating adequate sample collection and the correct functioning of the procedure.

2. HYPOTHESIS AND OBJECTIVES

Eldine has collected information from thousands of patients that wanted a diagnostic test during the fifth and sixth waves of the pandemic. The health protocol for the sixth wave recommended the end of the lockdown and the return of the workers to the workplace at 7 days after the first positive SARS-CoV-2 detection test. However, it has been observed that many people that came for a confirmation of negativity test at 7 days during the sixth wave were still positive, creating the hypothesis that more days might have been necessary to negativize.

Therefore, the **main objective** of this work is to study the behavior of SARS-CoV-2 throughout the fifth and sixth waves of pandemic in a cohort of 81 patients from Eldine Patología (13 from the fifth and 68 from the sixth wave) that had taken two or more RT-PCR tests in a maximum period of 15 days, of which at least the first one was positive, as well as to compare those results with the indications of the health protocols that the *Generalitat de Catalunya* published at those times.

Secondary objectives:

1. To collect and analyze data regarding RT-PCR positivity of the mentioned patients during the fifth and sixth waves.
2. To analyze the negativity (virus clearance) of those patients at 10 or 7 days after first positive RT-PCR result depending on whether they belong to the fifth or sixth wave group, respectively.
3. To analyze the virus behaviour in those patients that remain positive at 10 or 7 days, depending on whether they belong to the fifth or sixth wave group, respectively.
4. To confirm the positivity for the omicron variant in a subset of SARS-CoV-2 positive samples from the sixth wave by RT-PCR.
5. To clinically interpret the whole set of results obtained.

3. MATERIAL AND METHODS

3.1 Study Subjects

In the present study, from a total of 1816 patients from the fifth wave and 2408 patients from the sixth wave who went to Eldine Patología to get tested (4224 patients in total), 81 patients were included (13 from the fifth and 68 from the sixth wave). In Spain, the fifth wave was defined as the period of time between June 16th and August 14th, 2021, and the sixth wave as the period between December 1st, 2021, and January 31st, 2022. Those patients had taken two or more RT-PCR tests in a maximum period of 15 days, of which at least the first one was positive. The results, Ct values, clinical data and demographic data (age and sex) of these patients were analized for this study.

3.2 Sample Collection and Handling

Nasopharyngeal exudate samples were collected by using a swab (Figure 11) that met the European Community directive requirements for medical devices (propylene handle and viscose head). Samples were collected from nasopharynx because there are high levels of viral reservoirs there. At the time of extracting the sample, Personal Protective Equipment was used, which consists in a biosecurity lab coat, gloves, a FFP2 mask and safety lab goggles. Each patient was seated at a chair and their head was tilted at an angle between 45º and 75º. The swab was introduced through each nostril following a parallel trajectory to the palate and with a slight rotation. Once the optimal zone is reached, the swab was taken out, while keeping

the rotation. After that, samples were manipulated into a type II biosecurity Telstar cabin (Bio-II-A), with the necessary sterile conditions at all times.

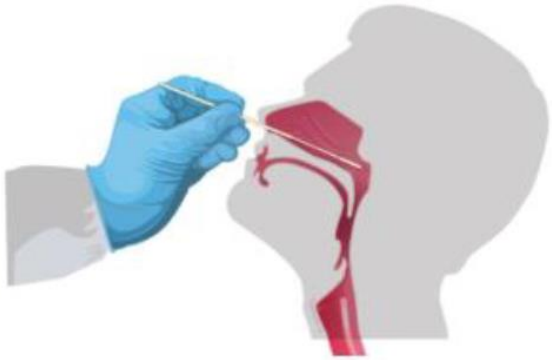

Figure 11: Sample collection using a swab Source: Asociación Española de Biopatología Médica (AEBM)-Medicina de Laboratorio

3.3 SARS-CoV-2 genome detection

SARS-CoV-2 genome detection was performed by quantitative RT-PCR by using the artus ® SARS-CoV-2 Prep&Amp UM Kit in a Rotor-Gene Q (RGQ) MDx thermocycler, both from Qiagen (Hilden, Germany). This is a one-step kit that does not require previous nucleic acids extraction and that contains TaqMan primers for detecting two different regions (72pb and 67pb) from the N gene of the SARS-CoV-2. Furthermore, it also contains primers for detecting RNAse P as human sample control whose detection should be positive in any case, ensuring an adequate sample collection. For the Omicron variant detection, primers detecting the K417N mutation in the S1 region of RBD and the deletion HV 69/70 in the S gene were used.

Briefly, samples should be first prepared by introducing the tip of the swab in 600µL of 0.5% NaCl sterile solution followed by 10-20” vortex, allowing attached cells to detach from the swab. Then 8µL of saline solution containing human cells was mixed with 2µL of a provided lysis buffer and incubated for 2 minutes at RT. On the other hand, the reaction mix was prepared by mixing 6.25µL of master mix containing enzymes with 8.75µL of primers per sample. Positive (synthetic SARS-CoV-2 sequence) and negative (nuclease-free water) controls were included in each determination. After 40 cycles of amplification, data relative to

positivity/negativity (cycle threshold, Ct) were stored. Cycling conditions can be consulted in Table 2.

Table 2. Cycling conditions for the RT-PCR test

Step	Time	Temperature
RT-step	10 min	50ºC
PCR initial heat activation	2 min	95ºC
2-step cycling (40 cycles)		
Denaturation	5 s	95ºC
Combined annealing/extension	30 s	58ºC

3.4 Statistical analysis

The analysis of the Ct values between groups was performed by using Microsoft Excel. Differences between the two groups were determined using Student's t-test (two-tailed, 95% confidence interval). A P-value <0.05 was considered statistically significant in all analyses.

4.RESULTS

4.1 Demographic data from study subjects

Demographic data from the study subjects are shown in Table 3. 13 patients were included in the fifth wave group, whereas 68 were included in the sixth wave group. The number of men and women were similar between groups, as well as the percentage of people that reported symptoms. However, it should be taken into account that some patients don't declare if they have symptoms. In the fifth wave, 30.8% of the patients were women and 69.2% were men, with an age average of 27±8 years. Out of these patients, 46.2% reported symptoms. For the sixth wave, 36.8% of the patients were female and 63.2% were male, with an age average of 36,6±12,4 years. Out of these patients, 41.2% had symptoms. Regarding the mean Ct (cycle threshold) between groups, there was a significant decrease in the sixth wave group (p=0,009).

Table 3. Demographic data of the studied population.

	Fifth wave (N=13)	Sixth wave (N=68)
Women	4 (30.8%)	25 (36.8%)
Men	9 (69.2%)	43 (63.2%)
Age average	27 ± 8	36,6 ±12,4
Patients with symptoms	6 (46.2%)	28 (41.2%)
Mean Ct**	28,7±6	25±4,7*
Main SARS-CoV-2 variant	Delta	Omicron

*p<0,05

**Ct: Cycle threshold

4.2 RT-PCR positivity before recommended quarantine time

First, it was studied how many patients had a second RT-PCR before the minimum quarantine time passed (10 days in the fifth wave and 7 in the sixth) and which are the results. Table 4 indicates that amongst the patients from the fifth wave, 4 had another test before 10 days but only one of them was still positive (25%) with a Ct of 37. In the patients from the sixth wave there were 29 who had a second test before 7 days and 7 of them were still positive at that time (24,1%) with a Ct average of 27±4. The percentage of positive patients between waves is very similar, indicating that the variants follow a similar behavior before the recommended quarantine times.

Table 4. Second PCR test before 10 and 7 days in the fifth and sixth wave, respectively

	Fifth wave (before 10 days)	Sixth wave (before 7 days)
Total	4	29
Positive	1 (25%)	7 (24,1%)
Mean Ct	37	27±4
Negative	3 (75%)	22 (75,9%)

4.3 RT-PCR positivity at the end of recommended quarantine time

Next, virus clearance in the patients that showed a positive RT-PCR after 10 days (fifth wave group) or 7 days (sixth wave group) was explored, since those are the days that the official health protocols indicate for them to get out of confinement. The results obtained are shown in Table 5. For the first group only 4 patients had an additional test at exactly 10 days after the first RT-PCR positive result, while 9 in the second group at exactly 7 days. 100% of the patients of the fifth wave were negative at 10 days, while 77.8% of the sixth wave were negative at 7 days, which means that 22.2% of those patients remained positive at the time that the health protocol indicates deconfinement, with a mean Ct of 28,6±3,5.

Table 5. Second PCR after 10 and 7 days in the fifth and sixth wave, respectively

	Fifth wave (10 days)	Sixth wave (7 days)
Total	4	9
Positive	0	2 (22.2%)
Mean Ct	0	28,6±3,5
Negative	4 (100%)	7 (77.8%)

4.4 RT-PCR positivity after the recommended quarantine time.

Patients that were positive between the end of the minimum quarantine time and a maximum of 15 days after the first positive RT-PCR were also studied. As it can be observed in Table 6, 5 patients did a test after 10 days in the fifth wave and none of them were positive. However, in the sixth wave 33 patients did a test after 7 days and 7 of them were still positive (21,2%), with a mean Ct of 26,8±2,6, results very similar to those from the sixth wave group in the previous section. Moreover, it is worth noting that 5 of those 7 patients were tested at 8-10 days. According to the previous section, this data suggests that 7 days of quarantine in the sixth wave might be insufficient. For instance, if a 10-day quarantine had been followed in the sixth wave, less than a third (6%) of the patients would still be positive at that time.

Table 6. Second PCR between 10-15 and 7-15 days in the fifth and sixth waves, respectively

	Fifth wave (between 10 and 15 days)	Sixth wave (between 7 and 15 days)
Total	5	33
Positive	0	7 (21,2%)
Mean Ct	0	26,8±2,6
Negative	5 (100%)	26 (78,8%)

4.5 Patients with a third RT-PCR test within 15 days

Finally, those patients who had a third RT-PCR within the next 15 days after the first positive RT-PCR were studied. The results can be seen in Table 7. There is no data for the fifth wave because no patients took a third test at that period. However, in the sixth wave 6 people took a third test within that time, from which only one of them was still positive with a Ct value of 27,2. These results indicate that in some cases the COVID19 infection in the sixth wave can be very durable, reinforcing the idea that 7 days of recommended quarantine for the sixth wave might be insufficient in some cases, to negativize.

Table 7. Third PCR in 15 days in the fifth and sixth waves

	Fifth wave (third PCR in 15 days)	Sixth wave (third PCR in 15 days)
Total	0	6
Positive	0	1 (16,7%)
Ct	0	27,2
Negative	0	5 (83,3%)

4.6 Omicron variant detection in patients of the sixth wave

To further confirm that the time ranges for the fifth and sixth waves (June 16th to August 14th and December 1th to January 31st respectively), were the Delta or the Omicron variant predominate respectively, are suitable, an RT-PCR was performed in a subset of 20 positive patients from the fifth (n=10) or sixth (n=10) wave groups by using specific primers for the K417N and Del HV 69/70 mutations, which are characteristic of the Omicron variant.

The results showed that 6 patients (60%) from the sixth wave group were positive for the Omicron mutations, while none of the patients from the fifth group displayed positivity (Table 8 and Figure 12). Since no patients from the fifth wave were positive for the Omicron variant and during the sixth wave the Omicron variant lived together with the Delta variant, this data might indicate that the time frames selected for the fifth and sixth waves are adequate and reflect the epidemiological data described. Moreover, this data reinforces the concerns about whether the official health protocol for the sixth wave and its quarantine recommendation was insufficient, given that during this wave the Delta variant (or, at least, a different variant) was also present.

Table 8. Omicron detection for the fifth and sixth waves

	Fifth wave	Sixth wave
Total	10	10
Positive for Omicron	0	6 (60%)
Negative for Omicron	10 (100%)	4 (40%)
Ct	0	31,3±1,8

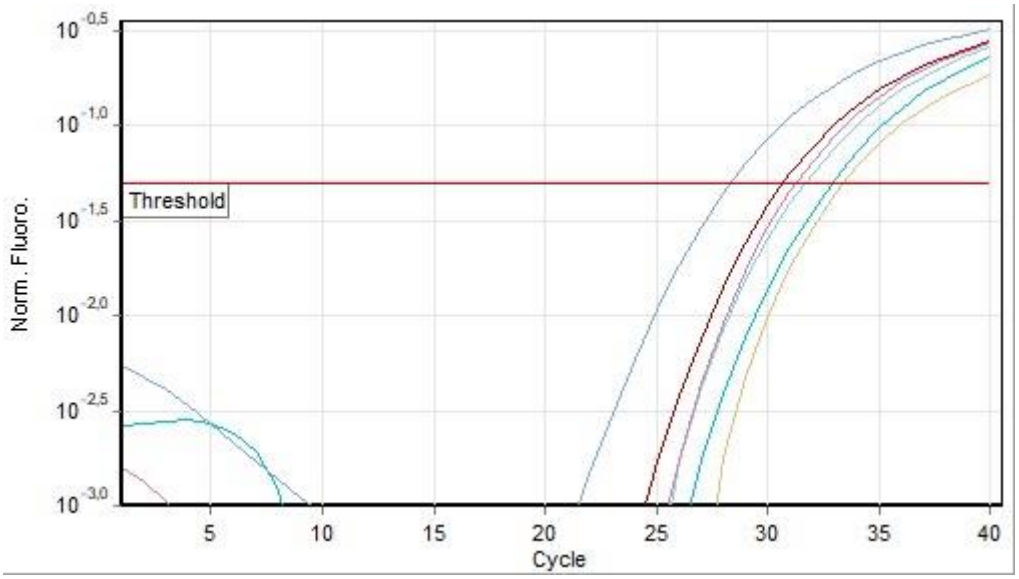

Figure 12: Detection of the mutations K417N and Del HV 69/70 from the Omicron variant. N=10 from the fifth wave and N=10 from the sixth wave. This figure displays the amplification of six patients from the sixth wave group.

5. DISCUSSION

In the current situation the pandemic has left us, it is of utmost importance to understand the characteristics and behavior of each new variant of SARS-CoV-2 to see its severity and how long it takes the body to eliminate it.

As mentioned before, the main objective of this study was to study the behavior of SARS-CoV-2 throughout the fifth and sixth waves of the pandemic as well as to compare those results with the indications of the health protocols that the Generalitat de Catalunya published at those times. It could be observed that at exactly 10 and 7 days after the first positive RT-PCR results for the fifth and sixth wave groups, respectively, 22.2% of the patients from the sixth wave were still positive, whereas all patients from the fifth group were negative. This could mean that the recommended quarantine time for the fifth wave was sufficient, while in the sixth wave it fell slightly short. Because of these results, up to 15 days after the first positive RT-PCR were studied and it was found that 7 patients from the sixth wave were still positive, and 5 of them were positive before the 10th day, which are the days recommended for quarantine in the fifth wave. This means that only 6% of the patients from the sixth wave were still positive after 10 days suggesting that it might have been better to leave the recommended quarantine time at 10 days, instead of reducing it to 7, according to viral clearance time.

RT-PCR tests were performed with samples from the fifth and sixth wave, and confirmed that the Omicron variant was the dominant variant in the sixth wave, but it was also seen that another variant was still very present in this wave (probably Delta, as it will be valued from now on). This means that the restrictions from the protocol of the fifth wave have to be taken into consideration because 40% of the samples from the time period of the sixth wave were not Omicron, and were probably Delta.

Some works have studied the infectiousness and durability of the Delta and Omicron variants. Wang et al. described that Delta variant displayed lower Ct (higher viral load) than the wild-type SARS-CoV-2 regardless of severity. In this study, the median of Cts was 20,6 for the Delta variant and 34,0 for the wild-type.[20] In another study, Park et al. the initial viral loads were not significantly different between the Delta group and the non-Delta group; however, during the late course of disease (day 3 to day 10 after symptom onset), the viral load was significantly higher in the Delta group than in the non-Delta group.[21] Regarding Omicron, several studies, Matic et al. and Fall et al., have been made and they all show that the viral load (Ct values) of both Delta and Omicron do not differ notably.[23,24] Here it must be pointed out that in this study, patients from the fifth wave (where Delta is the dominant variant)

had a slightly higher Ct than those in the sixth wave (where Omicron is the dominant variant). This could be because, even if Omicron is the dominant variant in the sixth wave, there is a mixture of both Delta and Omicron as it has been seen before, whereas in the fifth wave there is a mixture between Delta and the wild type variants mentioned earlier who have a higher Ct value.

However, the decline rates of the viral load is what's more interesting for this work. One study, Singanayagam et al., showed that for the viral load of Delta to go from peak value down to an undetectable value, a 10 day period was required. In another study, Shen et al., they studied the decline rates of the viral load for Omicron in two different groups of people and found that it was 9,9 days for one group and 11,1 for the other.[25] If this is compared to the data from the Delta variant, it can observed that they both have a very similar decline rate, meaning that the recommended quarantine time should also be similar.

Throughout Spain, the recommended quarantine time was reduced to 7 days during the sixth wave. Other European countries followed similar protocols with slight differences. For instance, in France, the neighboring country, the 10 days of recommended quarantine ended at the end of the year 2021, the same as in Catalonia.[26,27] At this point, only people who were fully vaccinated had a 7 days quarantine time, after which they didn't need a negative test to finish their quarantine. If you aren't fully vaccinated you still have to do the 10 days of quarantine. Up until today, in France, these measures are still in use.[28]

6. LIMITATIONS

This study has several limitations that have to be considered:

1. The number of patients from the fifth wave (n=13) wasn't enough to be able to fully understand the behavior of the Delta variant.
2. The sample size between both waves is vastly different being 13 patients in the fifth and 68 in the sixth wave.
3. The patients didn't wait the same number of days between the first test and the second meaning that the time between tests varies from one patient to another.
4. Only the Omicron variant tried to be detected in both waves (with no presence in the fifth wave and a 60% presence in the patients from the sixth wave). The rest was valued as Delta but it could have been other variants.

7. CONCLUSIONS

From this research the following conclusions can be obtained:

- Less than 10 days of quarantine in the fifth wave and less than 7 days in the sixth wave were insufficient since some of the patients were still positive for SARS-CoV-2.

- Delta and Omicron variants behave very similarly regarding virus clearance, being the average time around 10 days after symptoms appear. However, there are some patients who take longer to eliminate the virus.

- The protocol for the fifth wave, where 10 days of quarantine were recommended, was suitable, since after this time all patients were negative.

- The protocol for the sixth wave, where 7 days of quarantine were recommended, was not sufficient for the viral clearance, since after this time 21,2% of the patients were still positive.

- Omicron is not the only variant in the sixth wave.

- Increasing the quarantine time for the sixth wave from 7 to 10 days decreased the positive rates from 21.2% to 6%. Therefore, a minimum of 10 days of confinement after a positive direct SARS-CoV-2 detection test, regardless of the variant for the viral clearance, is recommended.

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