UNIVERSIDADE Đ COIMBRA

Victor Leonel Vieira Ferreira

SARS-COV-2 DETECTION METHODS: WHERE ARE WE NOW?

Dissertação no âmbito do Mestrado em Biotecnologia Farmacêutica, orientada pelo Doutor Hugo João Marques Prazeres e pelo Professor Doutor Luís de Almeida e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

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"It had long since come to my attention that people of accomplishment rarely sat back and let things happen to them. They went out and happened to things."

Leonardo Da Vinci

Abstract

SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) is a coronavirus (CoVs) that belongs to the family *Coronaviridae* and the genus *Betacoronavirus*, considered to be the largest group of viruses causing respiratory and gastrointestinal infections in humans and animals. The first cases of the disease caused by the new member of CoVs in humans (Coronavirus Disease 2019 (COVID-19)) appeared in December 2019 in the city of Wuhan, in China. SARS-CoV-2 is a virus with a higher human infection capacity, compared to other CoVs of the same genus already discovered. In a short time, this virus caused thousands of infections and deaths in China, and consequently, has been propagating throughout the world, being recognized a pandemic by the World Health Organization (WHO) on March of 2020. Owing to the lack of specific treatments, vaccines and screening methods, COVID-19 has become a major public health problem.

However, detection methods currently used are based on invasive methods, namely the use of swabs through the nose or mouth to scrap cells from the nasopharynx or oropharynx, respectively. In recent years, the interest for minimally invasive procedures has grown in medicine, leading to greater success in the biological fluids-based evaluation, for several diseases and infections. Aside from the nasopharynx and oropharynx, the SARS-CoV-2 virus is found mostly in the lower respiratory tract. SARS-CoV-2 detection in saliva, stool and urine has been reported, and to this date, no data were disclosed about which biological sample allows higher sensitivity. Also, it is needed to establish the methods most suited for SARS-CoV-2 detection in asymptomatic individuals and/or at a presymptomatic stage, which are more suitable for population-wide studies.

In this work, we review the key features of SARS-CoV-2 and COVID-19 and analyse the testing methods available to detect SARS-CoV-2 presence.

Keywords: SARS-CoV-2, COVID-19, transmission, viral RNA, detection methods.

Resumo

SARS-CoV-2 (Síndrome Respiratória Aguda Grave Coronavírus 2) é um coronavírus (CoVs) que pertence à família *Coronaviridae* e ao género *Betacoronavirus*, sendo considerado o maior grupo de vírus que causam infeções respiratórias e gastrointestinais em humanos e animais. Os primeiros casos da doença provocada pelo novo membro dos CoVs em humanos (Doença causada pelo Coronavírus 2019 (COVID-19)) surgiram em dezembro de 2019 na cidade de Wuhan, na China. SARS-CoV-2 é um vírus com uma maior capacidade de infeção em humanos, tendo por comparação outros CoVs do mesmo género já descobertos. Num curto período de tempo, este vírus causou milhares de infeções e mortes na China, e consequentemente, disseminou-se pelo mundo, sendo declarada a situação pandémica pela Organização Mundial da Saúde em março de 2020. Por falta de tratamentos específicos, vacinas e testes de deteção, a COVID-19 é um grande problema de saúde pública.

Contudo, os métodos de deteção atualmente utilizados baseiam-se em métodos invasivos, nomeadamente a aplicação de zaragatoas no nariz ou na boca para retirar células da nasofaringe ou orofaringe, respetivamente. Nos últimos anos, o interesse por procedimentos minimamente invasivos tem crescido na medicina, o que levou a um maior sucesso das análises baseadas em fluidos biológicos, para diversas doenças e infeções. Ademais da nasofaringe e orofaringe, o vírus SARS-CoV-2 encontra-se principalmente no trato respiratório inferior. A deteção do vírus na saliva, fezes e urina tem sido reportada e, até o momento, não foi relatada nenhuma informação sobre qual amostra biológica permite maior sensibilidade. Além disso, é necessário estabelecer métodos mais adequados para a deteção de SARS-CoV-2 em indivíduos assintomáticos e/ou em fase pré-sintomática, os quais são mais adequados para estudos da população em geral.

Neste trabalho reportamos as principais características do SARS-CoV-2 e da COVID-19 e analisamos os testes disponíveis para a deteção do SARS-CoV-2.

Palavras-chave: SARS-CoV-2, COVID-19, transmissão, RNA viral, métodos de deteção.

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List of Abbreviations

ACE2 – Angiotensin-converting enzyme 2

Bat-CoV-RaTG13 – Coronavirus previously identified in a bat species from the Yunnan region

CDC – Center for disease control

CoV – Coronavirus

COVID-19 – Coronavirus disease 2019

CRISPR – Clustered regularly interspaced short palindromic repeats

CSG – *Coronaviridae* study group

CT – Computed tomography

Ct – Cycle threshold

DNA – Deoxyribonucleic acid

EBA – Exhaled breath aerosols

EBC – Exhaled breath condensate

ELISA – Enzyme-linked immunosorbent assay test

E protein – Small envelope protein

ERGIC – Endoplasmic reticulum-Golgi compartment

IgG – Immunoglobulin G

IgM – Immunoglobulin M

ICTV – International committee on taxonomy of viruses

ICU – Intensive care unit

K_d – Dissociation constant

LFIA – Lateral flow immunoassay test

LAMP – Loop-mediated isothermal amplification

MERS-CoV – Middle east respiratory syndrome coronavirus

mAb – Monoclonal antibody

M protein – Matrix protein

NGS – Next generation sequencing

N protein – Nucleocapsid protein

NSP – Non-structural protein

ORF – Open reading frame

Pangolin-CoV – Coronavirus similar to severe acute respiratory syndrome coronavirus 2, found in dead pangolins from Malaysia

PCR – Polymerase chain reaction

R_0 – Basic number of reproduction

RBD – Receptor binding domain

R_e – Effective number of reproduction

RdRp – Ribonucleic acid-dependent ribonucleic acid polymerase

RNA – Ribonucleic acid

RT-PCR – Reverse transcription polymerase chain reaction test

RT-RPA – Reverse transcriptase and recombinase polymerase test

R_0 – Basic number of reproduction

SARS-CoV – Severe acute respiratory syndrome-associated coronavirus

SARS-CoV-2 – Severe acute respiratory syndrome coronavirus 2

S protein – Spike glycoprotein

SVN – Serum virus neutralization assay

TMPRSS2 – Transmembrane serine protease type II

USA – United States of America

VOC – Volatile organic compound

WHO – World health organization

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1. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

1.1. History and Origin of infection

CoVs were first denominated as a new respiratory tract virus (1) when they were observed in samples taken from patients who demonstrated signs and symptoms of a respiratory tract infection in 1962 (1, 2) and are generally considered non-deadly pathogens for humans, causing only 15.0% of common mild colds (2-4). However, over time, CoVs with a high pathogenic capacity compared to the ones who were previously found in humans, for instance, in the case of the outbreak of severe acute respiratory syndrome (SARS) that appeared on the Guangdong region of China in late 2002 (1-4). This outbreak caused by SARS-associated coronavirus (SARS-CoV), which began in November 2002 in China and ended in August 2003, spread throughout the world, affecting 32 countries, mainly on the Asian continent, with 8,422 cases and 916 deaths in total (1, 2).

In December 2019, a growing group of patients with potentially severe pneumonia was confirmed to have been infected by a new CoV, which was not previously observed in humans, in the city of Wuhan, capital of the Hubei region of China (1-5). This new strain of coronavirus was initially called 2019 novel CoV and the disease it causes designated by COVID-19 (1, 3, 5). Later, in February of 2020, the WHO renamed this new CoV to SARS-CoV-2, since the demonstrated pathogenic characteristics, transmission mechanisms and symptoms are highly similar to SARS-CoV (1, 3). Since December 31, 2020, considered the zero day of the pandemic, until February 27, 2020, more than 80,000 infectious and more than 2500 deaths had been registered, affecting 47 countries worldwide (4). Currently, after 274 days of day zero (October 4, 2020), the numbers registered are already more than 34 million infectious and more than 1 million deaths, affecting 216 countries and territories (6). In Portugal, the arrival of the pandemic by COVID-19 became official, when the first two positive cases emerged on March 2, 2020 (7). Currently, after 212 days (October 4, 2020) since the first cases, accounted for more than 78,000 infectious and more than 1900 deaths (6).

These and other important events about the emergence of this new outbreak by SARS-CoV-2 are represented, in a more detailed way, a timeline found in **Figure 1**.

SARS-CoV-2 shown a greater capability to infect humans than other CoVs previously discovered, namely SARS-CoV and middle east respiratory syndrome coronavirus (MERS-CoV) (1). The latter originated other outbreak in 2012, beginning in Saudi Arabia and soon has spread to 27 other countries, resulting in 2494 cases of infection and 868 deaths (2-4). Several factors contributed to the rapid spread of SARS-CoV-2, among which are the large

population density of Wuhan, with more than 11 million inhabitants, and the fact that this city is a transportation hub, which increases the likelihood of transmission of the virus between people, as well as the exportation of cases to other regions and countries (1).

That way, the recent SARS-CoV-2 virus, causer of COVID-19 disease has become the third outbreak of CoV recorded human history (3), which led to the implementation of social containment measures, closure of companies and commercial establishments, among others, threatening the economy of all countries with confirmed and suspected cases of infection (4), which continues to grow dramatically (2).
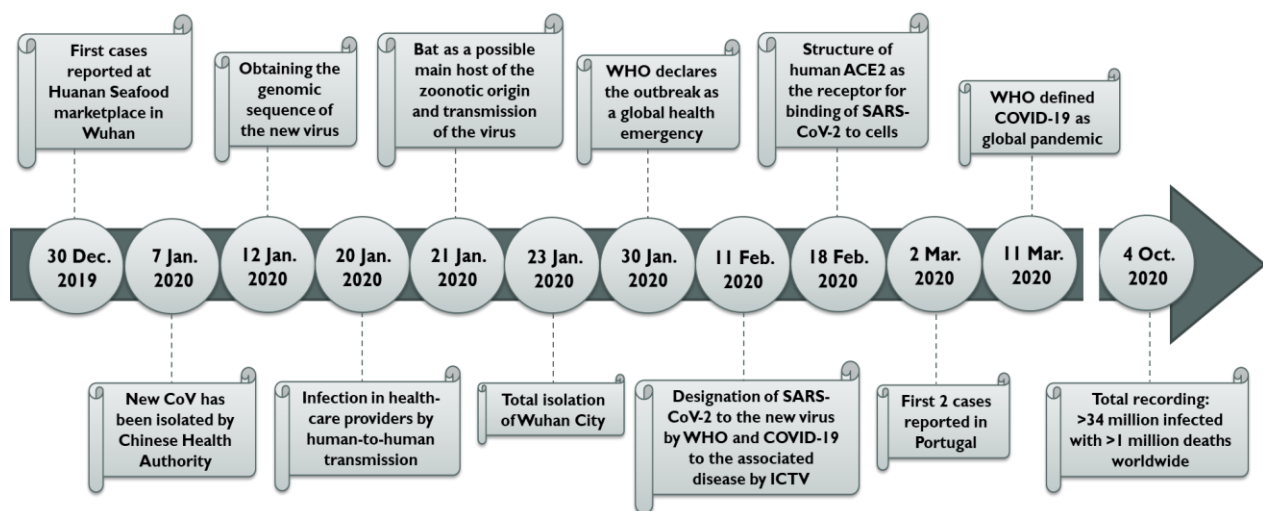
Figure 1 - Timeline of the major events the emergence of this new outbreak by SARS-CoV-2. Adapted from (3, 4, 8, 9).

Legend: ACE2 – angiotensin-converting enzyme 2, CoV – Coronavirus, COVID-19 – coronavirus disease 2019, ICTV – International Committee on Taxonomy of Viruses, SARS-CoV-2 – severe acute respiratory syndrome coronavirus 2, WHO – World Health Organization.

1.2. Etiology - Virological characteristics of SARS-CoV-2

1.2.1. Classification, Genome and Virion structure

- Classification

From epithelial cells belonging to the airways of infected patients it was possible to isolate this new CoV, provisionally designated from 2019 novel CoV. Later, the *Coronaviridae* study group, which is part of the International Committee on Taxonomy of Viruses (ICTV), who is responsible for the classification of viruses and their taxonomic nomenclature in the *Coronaviridae* family, found that this new CoV has a link to the SARS-CoV virus, both of which were included in a category of species called *Coronavirus associated with severe acute respiratory syndrome* and on February 11, 2020, it was designated SARS-CoV-2 (10, 11).

CoVs, such as SARS-CoV-2, represent simple chain ribonucleic acid (RNA) enveloped viruses with positive and non-segmented meaning, whose sizes range from 26 to 32 kilobases. As for phylogenetics and taxonomic nomenclature, according to ICTV, these are part of the subfamily *Coronavirinae,* family *Coronaviridae* and order *Nidovirales* (10-14). According to the serotype character (equal type and number of antigens on the surface of the virus, within the same species) and genomic, the subfamily *Coronavirinae* presents a separation into four important genera corresponding to *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*. Of the genera mentioned, the first two tend to infect particularly mammals, while gammacoronaviruses primarily infect bird species, and deltacoronaviruses infect both species (10, 13, 15). These CoVs are responsible for causing especially diseases in the respiratory and gastrointestinal, hepatic and neurological tracts, Six types of disease-causing CoVs were previously identified, covering HCoV-NL63 and HCoV-229E, which are part of the genus *Alphacoronavirus*, those of the genus *Betacoronavirus*, HCoV-OC43, HCoVHKU1, SARS-CoV, MERS-CoV and recently the SARS-CoV-2 itself (10, 14-17). Four of these species of CoVs, 229E, OC43, NL63 and HKU1 are commonly known to cause common cold symptoms in immunocompetent subjects, while strains of SARS-CoV and MERS-CoV were responsible for two previously mentioned worldwide outbreaks, which occurred in 2002 and 2012, respectively, causing more severe respiratory symptoms in patients and many deaths (16).

- Genome

The CoVs have a genome size of 26 and 32 kilobases, where a diverse number (6 to 11) of Open Reading Frames (ORFs) are inserted (15, 17, 18). The first ORF constitutes about 67.0% of the entire genome of the virus, encoding 16 non-structural proteins (NSPs), while the remaining ORFs encode the virus's accessory and structural proteins (15, 19). Of these structural proteins, there are four main proteins called spike glycoprotein (S protein), the small envelope protein (E protein), the matrix protein (M protein) and, finally, the nucleocapsid protein (N protein) (13, 15, 20). The S protein plays an important role in the binding, fusion and entry of the virus by the host cell receptors and, in turn, they represent essential inducers of neutralizing antibodies, which allow to the evaluate the host's tropism and its potential of transmitting the virus, through the binding with the receptor and the membrane fusion of the host cells (13, 15, 20, 21). The S protein that constitutes the CoVs is functionally divided by domain S1, in charge of binding to the host receptor and domain S2, responsible for the fusion to the cell membrane of the host's cells (20, 21).

In SARS-CoV-2, 14 ORFs were identified throughout its genome, capable of encoding 27 proteins. As for the orf1ab and orf1a genes, which are positioned in terminal 5' of the virus genome, they are responsible for encoding the proteins pp1ab and pp1a, respectively. Together, constitute 15 (NSPs), namely nsp1 to nsp10 and nsp12 to nsp16. In terminal 3', the genome of SARS-CoV-2 is composed of eight accessory proteins (3a, 3b, p6, 7a, 7b, 8b, 9b and orf14) and four primordial structural proteins (S, E, M and N), indicated above, being possible to observe in **Figure 2B** (15).

Regarding a phylogenetic analysis of that same study, from a phylogenetic tree based on the whole genome sequencing, it was found that SARS-CoV-2 was positioned parallel to the CoVs present in bats like SARS-like bat CoVs whereas the SARS-CoV descended from this last lineage, showing that SARS-CoV-2 was closer to the CoVs present in bats, compared to the SARS-CoV. The greatest similarity was found between SARS-like bat CoVs and SARS-CoV-2. In comparison, SARS-CoV-2 and MERS-CoV demonstrated to be genetically distant and less associated. (15).

Although the phylogenetic analysis, in general, clearly shows that SARS-CoV-2 is more related to SARS-like bat CoVs, some genetic similarities with SARS-CoVs are notorious. For example, whereas for proteins pp1ab, pp1a, E, M, accessory protein 7a, N protein genes and S protein gene, there is a greater genetic similarity between SARS-CoV-2 and SARS-like bat CoVs, SARS-CoV-2 accessory genes 3a and 8b are more similar to those of SARS-CoVs. Taking into account the genetic similarities between these CoVs, further analyses on differences in amino acid substitutions in different proteins could clarify the structural and functional differences between them, and in what sense they influence the functioning and pathogenesis of SARS-CoV-2. For instance, in the referred study, comparing the amino acid sequences of SARS-CoV-2 with those of SARS-CoV-2 and SARS-like bat CoVs, it was possible to evidence more than 300 amino acid substitutions. Nevertheless, due to the lack of understanding that exists about this new virus, it was not possible to justify this considerable number of amino acid substitutions. However, if these discrepancies can influence host tropism or the transmission capacity of SARS-CoV-2 in relation to SARS-CoV, for instance, its research would become crucial in the near future.

- Virion structure

The concept "coronavirus" highlights the appearance of CoV virions when they are identified by electron microscopy, being possible to identify spike projections of the virus membrane, giving the appearance of a crown, or *corona* in Latin (14).

Therefore, as mentioned above, the SARS-CoV-2, belonging to the class of CoVs virions, (**Figure 2A**) has a diameter ranging from 60 to140 nm and different peaks to the surface with lengths between 9-12 nm (22). Its structure represents a transmembrane protein, whose molecular weight is approximately 150-180 kDa and location is on the outside of the surface of the virus (18, 23-25). This S protein has a homo-trimeric form protruding from the viral surface, which favors the interaction of enveloped viruses with host cells, through affinity with the angiotensin-converting enzyme 2 (ACE2) (18, 24-26). The trimeric S glycoprotein is also cleaved in two subunits called S1 and S2, this cleavage may occur through one or more proteases present in the host, from furin, trypsin, cathepsin proteases, transmembrane serine protease type II (TMPRSS2), TMPRSS4 or even by a human airway trypsin-like protease (27-30). In the case of SARS-CoV and MERS-CoV, they use the CTD to bind to the host cell receptor (31-33).

At the limit between subunits S1 and S2 of SARS-CoV-2 S protein, it was also possible to identify a polybasic furin cleavage site (RRAR amino acid sequence), by including 12 nucleotides in its gene, resulting in the previous addition, along the site of three glycans linked to oxygen atom of serine or threonine residues of protein (S673, T678 and S686), particular to SARS-CoV-2, since a proline residue is inserted in that site, and the sequence is PRRAR (24, 34). This cleavage site is made throughout the virus biosynthesis process in an effective manner by furin and other possible proteases (trypsin and cathepsin) (24, 34, 35).

That said it is known that the entry of SARS-CoV into the host cells occurs by the same receptor of these cells in SARS-CoV-2 (36-38). This receptor, already mentioned, is ACE2 which despite recognizing some CoVs, its main physiological function in the body is the maturation of angiotensin. It represents a type I membrane protein, present in cells of various organs, such as the lungs, heart, kidneys and intestine (39-41). Therefore, a reduction in ACE2 expression is related to cardiovascular diseases (42, 43). The S1 subunit receptor binding domain (RBD) of the trimeric S protein will bind to the peptidase domain of the ACE2 receptor in the host cells, whose dissociation constant (K_d) determined was approximately 15.0 nM, causing viral infection (44-47). All these structures were possible to observe by cryostructures, through electron microscopy technique (44).

The N protein, represents the structural constituent of the CoV located in the zone of the endoplasmic reticulum-Golgi, interacting structurally with the genetic material of the virus. By being the Virus RNA-bound N protein, it implies its association with genetics processes such as the replication cycle and the response of host cells, during viral infections (48, 49). This same protein is also substantially phosphorylated and may be implied to cause structural changes that allow a higher affinity with viral RNA (18, 50).

In terms of M protein or the membrane or matrix, it is also another fundamental component in the CoVs, since it constitutes the most organized protein, at the structural level and has the function of defining the enveloped form of the virus. Thus, the M protein can bind to the remaining set of structural proteins of the virus. Regarding N protein, this interaction with M protein contributes to its stabilization and favors the end of the viral assembly process by stabilizing the N-RNA protein complex inside the internal virion in the host cell (49, 50).

Finally, the last constituent in the Structure of The CoV corresponds to the E protein or envelope, which is the smallest protein in the structure of SARS-CoV and responsible for producing and maturing this same virus (49, 50).

The high incidence and wide distribution of CoVs in animals, a considerable genetic variety and common recombination of their genomes, as well as an increase in human-animal interface activities, common infections between species and also accidental events of overflow, make it more likely that new CoVs will appear frequently in the human species (10, 16).

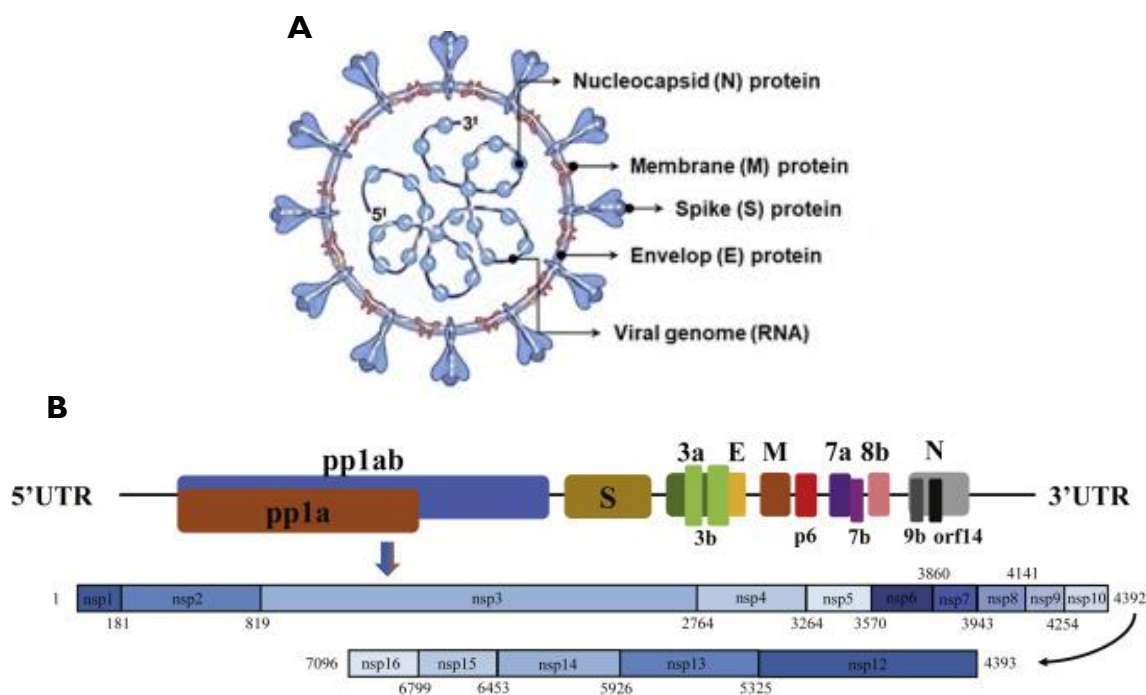**Figure 2 -** (A) The structure of SARS-CoV-2. Adapted from (51). (B) Genome constitution and organization of SARS-CoV-2 (IVDC-HB-01/2019 (HB01) strain). Adapted from (15).

Legend: E – envelope protein, M – matrix protein, N – nucleocapsid protein, NSP – non-structural protein, ORF – open reading frame, pp1a – polyprotein 1a, pp1b – polyprotein 1ab, RNA – ribonucleic acid, S – spike protein.

1.2.2. Life cycle of SARS-CoV-2

Viruses, usually in nanotechnology, can be associated with molecular nanomachines capable of controlling host cells, forcing them to create large amounts of copies of the virus itself (51, 52). Although the replication life cycle of viruses greatly diversifies, depending on the species and category of the virus, it comprises six elementary stages we must understand, which are divided into attachment, entry, uncoating, replication, maturation and release (51, 53).

Considering the CoVs belonging to the *order Nidovirales*, an infection by SARS-CoV-2 can be contracted from animals, as is the case of bats (50, 54). These types of viruses are able to enter the human body through the receptors of the ACE2 (50, 51, 54-56), present on the surface of cells of various organs, such as heart (myocardial cells), lungs (type II alveolar cells), kidneys (proximal tubular cells), liver (cholangiocytes), bladder (urothelial cells) and also in the gastrointestinal tract (upper and stratified epithelial cells of the esophagus and absorbent enterocytes), favoring viral entry in several target cells and thus potentiating Infection by SARS-CoV-2 (50, 51, 54, 56). This specific link shows the type of cell that can be infected by the virus and this event is called cell tropism (51, 53).

This method of entry of CoVs into host cells starts with the interaction and binding of S glycoprotein with the ACE2 receptor, being the type II pneumocytes present in the lungs one of the most representative places where ACE2 is expressed and thus represent the first stage of the virus life cycle, called **attachment** (50, 51, 54, 56). Some current studies have highlighted the crucial role of ACE2 receptor as a mediator in the entry of SARS-CoV-2 into host cells (24, 36, 38). One example was in HeLa cells that when expressing the ACE2 receptor on their surface, they were susceptible to a SARS-CoV-2 infection, however, cells that do not express that receptor are not infected (26, 36). The interaction occurs in the RBD zone of subunit S1 of S protein present in SARS-CoV-2, and the fragment is located between amino acid residues from position 331 to 524 of S protein and can be linked with high affinity to human ACE2 and bat, which indicates the importance of RBD as a crucial functional constituent in subunit S1 for the performance of this binding (24, 48, 50, 57). All these procedures of binding and entry of the virus are accompanied by the process of viral membrane fusion, together with the host cell membrane, corresponding to the second stage of the life cycle called **entry** (24, 50, 51).

At this stage there is the inclusion of the viral replication complex for the host cell cytoplasm (51, 53). For SARS-CoV and SARS-CoV-2, these shows similarities in biochemical interactions and pathogenesis. Regarding TMPRSS2, it is responsible for cleaving (between subunits S1 and S2) and activating S protein (38, 51), allowing to expose a fusion peptide present in subunit S2,

which is introduced into the cell membrane, starting the fusion process between the viral and cellular membranes (18, 51).

Following the fusion, TMPRSS2, which is located on the surface of the host cell, will cline and remove the ACE2 receptor and thus activate peak-like S proteins connected to the receptor (50, 54). This same activation of S proteins results in conformational modifications and allows the entry of the virus into host cells (50, 58). Therefore, the TMPRSS2 and ACE2 proteins correspond to crucial elements to the entry of SARS-CoV-2. According to one study, it was possible to identify that the oral epithelial cells, particularly goblet/secretory cells and ciliated cells, demonstrate the highest expression of the ACE2 receptor throughout the respiratory tract (50, 59). In addition, SARS-CoV-2 when entering host cells will later release its genetic material into the cytoplasm and then will be translated in the nuclei (50, 60).

In a third stage called **uncoating**, this genetic material released by the virus is the positive-sense mRNA that will be responsible for the direct translation into proteins essential to the virus, resulting in a new synthesis of the viral structure and NSPs (18, 50, 51, 60). Throughout its genome, already mentioned above, this virus has approximately 14 ORFs in which each one is responsible for encoding various proteins, from structural to non-structural, whose function is related to the survival of the virus itself, as well as its virulence capacity (50, 60).

Then, in the fourth stage called **replication**, the NSPs encoded by a replication gene, have the function of replicating the viral genome (18, 51). Thus, in this stage of transformation, the first genetic segments to be translated are those that encode non-structural polyproteins, which translate into ORF1a and ORF1b, in order to originate two large overlapping polyproteins, called pp1a and pp1ab, which provides a change of event, at the ribosomic level (15, 50, 60). These polyproteins are complemented with enzymes such as papain-like proteases and by an M pro-like serine protease, for example, chymotrypsin-like protease, and are encoded in nsp3 and nsp5 (18, 50, 61). Then, when the cleavage between pp1a and pp1ab occurs, they become NSPs of 1-10 and 12-16, respectively (15, 18, 50, 61). These NSPs have essential functions in many processes between viruses and host cells (18, 50, 61).

Several of the NSPs will later create a replicase-transcriptase complex in double-membrane vesicles, which correspond essentially to a set of RNA-dependent RNA polymerases (RdRp), together with subunits that include helicases. Furthermore, this complex expresses a model for endogenous viral input genome for negative-sense genes, both for the progeny genome, as well as subgenomic RNA corresponding to intermediate products and then transcription of positive-sense mRNAs, mediated primarily by RdRp (50, 60, 61).

Subsequently, in the fifth stage of maturation or assembly, these subgenomic proteins are transformed into structural and accessory proteins, such as the M, S and E proteins that

constitute the virus, and then isolated into the endoplasmic reticulum and then displaced to an intermediate compartment of the endoplasmic reticulum-Golgi compartment (ERGIC) (18, 50, 51, 60). However, the previously replicated genome program can directly attach the N protein to the nucleocapsid form and be directed to the ERGIC to produce mature virion. That said, this compartment will allow mature virions to meet with several other structural proteins and thus create small portfolio vesicles that will be exported to the outside of the cell, through the exocytosis process (18, 50, 51, 60), constituting the sixth and last stage of the replication life cycle of the virus called release.

In addition, in SARS-CoV-2, a new cleavage site similar to furin was identified in the peak protein. This cleavage site, which does not exist in SARS-CoV, is possibly associated with the virus exit of from the host cell and thus allows an effective spread of the virus throughout the human population (51, 62).

Therefore, the life cycle of SARS-CoV-2 can be schematized through **Figure 3**.

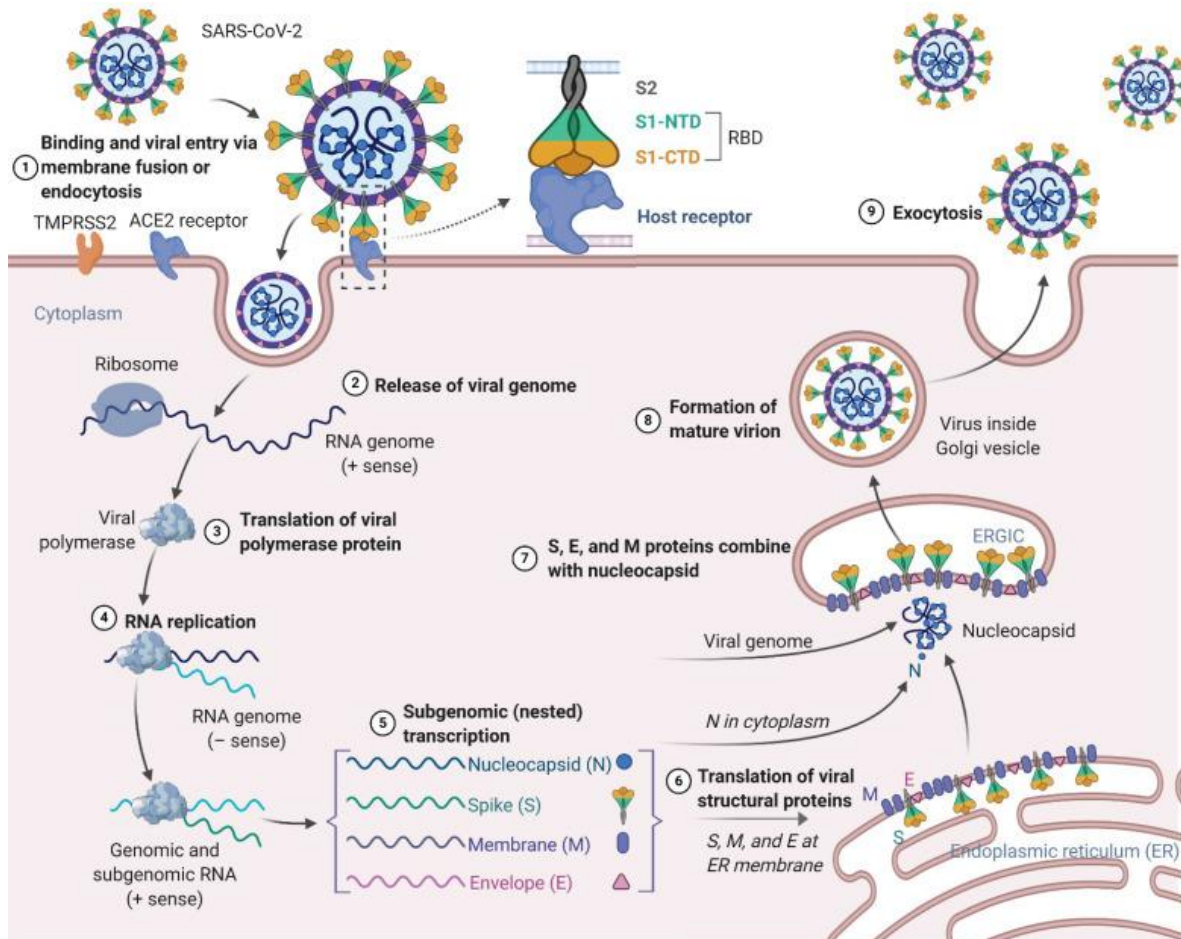**Figure 3 -** Representative diagram of the SARS-CoV-2 viral lifecycle. Adapted from (63).

Legend: ACE2 – angiotensin-converting enzyme 2, CTD – C-terminal domain, ER – endoplasmic reticulum, ERGIC – endoplasmic reticulum-Golgi compartment, NTD – N-terminal domain, RBD – receptor binding domain, RNA – ribonucleic acid, SARS-CoV-2 – severe acute respiratory syndrome coronavirus 2, TMPRSS2 – transmembrane serine protease type 2.

1.3. Epidemiology – Worldwide and in Portugal

Following the worsening of this new outbreak of SARS-CoV-2, it was declared as a pandemic on 11th March 2020 by the WHO in Geneva (64).

As on 3rd March 2020 90,870 cases of infection and 3,112 deaths (mortality rate of 3.4%) by SARS-CoV-2 have been confirmed (65, 66), which affected 73 countries (including Portugal) (66). The average age of patients reported varied between 41 and 57 years and the males represented the majority of patients, in a proportion ranging from 50.0 - 75.0% (65, 67, 68).

On 26th March 2020, 82,078 cases of COVID-19 infection and 3298 deaths were known in China, which resulted in a slightly higher mortality rate of around 4.0% (64).The Daily new cases of infection in this country peaked around February 12 (69) and since then the outbreak in China appears to be reduced due to a decrease in the number of cases daily diagnosed (64, 69). Initial studies showed that between 49.0 - 66.0% of all these cases had a history of contact with the seafood market in Huanan, where a variety of live wild animals were for sale and that was suggested as a possible origin of the outbreak of SARS-CoV-2 (65).

In spite of the decrease in active cases in China by late-February, the SARS-CoV-2 outbreak spread more rapidly in other countries (64). Based on WHO information until October 4, 2020, more than 34 million individuals are infected worldwide, were the most affected countries are the United States of America (USA) with more than 8,5 million cases, India with more than 7,9 million cases, and Brazil with more than 5,3 million cases. Contrarily, only 91,000 cases are reported from China (6). Nevertheless, these data do not indicate that the outbreak is not being fought in each country, where demanding measures have been adopted in order to track, detect, isolate and treat cases, which leads to the opportunity to contain the dissemination of the virus (64).

Although the whole population is susceptible to SARS-CoV-2 special attention and effort are needed to protect or decrease transmission in more vulnerable groups, such as children, health professionals, pregnant women, and the elderly (64). The symptoms and clinical conditions in infected patients were also analyzed in this same study reported on March 3, 2020. Data until this moment suggested that approximately 25.2 - 50.5% of people who were infected by this new CoV had one or more associated comorbidities such as diabetes, hypertension, chronic obstructive pulmonary disease, heart disease and malignancies (64, 65), probably due to having a weaker immune system, resulting in a faster progression of viral infection. Besides, this data suggested that SARS-CoV-2 has caused severe illness and death principally in the elderly, especially in the ones with pre-existing conditions such those

mentioned before. The probability of a child being infected with SARS-CoV-2 is similar to that of an adult, although it is found that the occurrence of severe symptoms is less recurrent in children (64).

Even so, measures such as closing schools are relevant measures to combat the spread of the virus, since, even asymptomatic, children are an important route of transmission of the new CoV (64).

In the case of pregnant women, although their proportion in confirmed cases is low, they represent a special group, once they are particularly vulnerable to respiratory pathogens and severe pneumonia, and the appearance of pneumonia during pregnancy can result in a variety of harmful obstetric conditions, such as premature rupture of membranes and premature delivery , intrauterine growth restriction and even neonatal death, among others, and thus provides high risks for both pregnant women and her unborn child (64). The mortality rate of pregnant women caused by respiratory pathogens is considerably higher than people. For example, the influenza virus pandemic in 1918 resulted in a mortality rate of about 2.6% for the entire general population but 37.0% for pregnant women (64). That said, pregnant women infected with SARS-CoV-2 should have particular medical attention regarding maternal and fetal monitoring and the comparison of the results with the results of diseases associated with other CoVs, such as SARS and MERS, could help the application of most appropriate treatments (such as early isolation, oxygen therapy, monitoring of fetal and uterine contractions, among others) (64).

The first data relating pregnancy to SARS-CoV-2 came from China but were greatly reduced, reporting just a small set of cases where pregnant women usually seemed to have mild symptoms of the disease. Regarding the vertical transmission of the virus, there is still insufficient evidence to prove it (70).

Regarding the national panorama, the first cases of infection by SARS-CoV-2 were detected on 2nd March 2020, and until April 22 there were 21,982 occurrences and 785 deaths of subjects infected by this new CoV. Public health surveillance data showed a considerable increase in the number of deaths in all existing causes of mortality in the period of March 2020 (10,096 deaths in total), compared to data from previous years (2018 and 2019) for the same time interval. Thus, an excess mortality of 2400 to 4000 deaths was confirmed in Portugal, which appears to be related with the increasing number of official COVID-19 deaths, and this excess of mortality was more present in older age groups (> 65 years), rather than in younger age groups (< 55 years), were on average, mortality was lower. This data presents three

possible causes that can relate to each other, namely, the COVID-19 itself, the COVID-19 not identified in patients who had it, and the reduction of access to health care. This last cause is showed with the substantial decrease in daily hospital emergency visits in more than 191,000, which may be possibly related with at least 1291 deaths (7)

However, at the begging of the pandemic, Portugal was able to act quickly with a complete restructure of the national healthcare system, in order to prepare it in the best possible way to optimize resources, and avoid its collapse (70, 71). Nevertheless, comparing the national situation with Europe is hard owing to the deficient data curation, the irregularities in the type and rate of data publication (for example lack of data on recovered patients, essential to determine the actual number of active cases), and the different approaches to stimulate COVID-19 testing (72).

In Europe, the first patient diagnosed with COVID-19 appeared on 24th January 2020 (73), and at this date are confirmed more than 9 million of COVID-19 cases . Also, the countries with the highest incidence of infected are France, Spain, and the United Kingdom which at this time were included in the top 9 list of countries with the most confirmed cases of SARS-CoV-2 infection (6). Therefore, all these data show that this new CoV has strong transmission routes and, in turn, high pathogenicity, which results in a great negative effect on global human health and on the economic progress of each country (74). Therefore, soon with the appropriate measures and rules, the COVID-19 pandemic may be controlled and even ceased with the emergence of an innovative vaccine.

1.3.1. Transmission of the coronavirus – zoonotic origin and intermediate host

- Possible zoonotic origin and intermediate host

Several recent studies support that SARS-CoV, MERS-CoV, and SARS-CoV-2, might have a common origin in the bats, however, the possible intermediate hosts might differ (36, 69, 75). Nevertheless, regarding the origin of SARS-CoV-2, three plausible theories were proposed that allowed to verify the improbability of SARS-CoV-2 to have arisen deliberately from laboratory manipulation of another CoV similar to SARS-CoV, and also of SARS-CoV-2 (34).

The first theory consists of a natural selection by an animal host before there is zoonotic transfer of the virus (34). Through bioinformatics analysis it was possible to demonstrate a 96.2% of homology between SARS-CoV-2 and the CoV previously identified in a bat species from the Yunnan region (Bat-CoV-RaTG13) genomic sequence (76-78), different of SARS-

CoV, which presented a homology in the genomic sequence of less than 80.0%. Although the Bat-CoV-RaTG13 virus is the most similar to SARS-CoV-2 with respect to its entire genome, the CoV similar to SARS-CoV-2, which was found in dead pangolins from Malaysia, at the beginning of the COVID-19 outbreak (Pangolin-CoV) is shown to be strongly similar to SARS-CoV-2 in the RBD area, which indicates that the SARS-CoV-2 spike protein, optimized for interaction with human ACE2, is a product of natural selection (34). Besides, SARS-CoV-2 has a polybasic cleavage site for furin in the spike protein to appropriate interaction with human ACE2, which means that the animal host would have to live within a high population density, allowing the natural selection of a virus more efficiently in its interaction with the human ACE2 receptor (34).

Then, the second proposed theory comprises the natural selection of the virus in humans, after having a zoonotic transfer (34). In this theory, it is likely that when there is the transmission of a progenitor of SARS-CoV-2 to humans, it may have acquired the genomic characteristics mentioned above, through adaptation in the course of unidentified transmissions between humans, even before the first cases reported in China at the end of December of 2019 (34, 78). Therefore, this panorama supports that there has been an unidentified transmission period in humans, between the incident of the initial zoonotic transfer and obtaining the polybasic cleavage site (34, 78).

Lastly, the third theory refers to a natural selection of the virus during the zoonotic transfer of an animal host to humans (34). Studies involving the passage of bat SARS-CoVs and/or other CoVs models for cell cultures demonstrated that SARS-CoV-2 is likely to have obtained the mutations in RBD, during the passage between cells in culture (34, 79, 80). Because Pangolin-CoV has RBDs practically similar to SARS-CoV-2, this information provides a much more convincing explanation of how SARS-CoV-2 obtained these characteristics, through recombination processes or mutations (34, 78).

Nevertheless, until now, all these theories about the zoonotic origin of the SARS-CoV-2 presented are impossible to prove or disprove, thus, their source is still to be determined. However, there is evidence to show that SARS-CoV-2 is not a virus deliberately manipulated in the laboratory (34). Based on the vast diversity of viruses present in wild animals and their constant evolution, especially in mammals, undoubtedly the most accessible and economical way to decrease the risk of future outbreaks, is to restrict our exposure to animal pathogens (78).

- Transmission of the virus between humans

Around the second half of January 2020, studies were conducted with groups of infected families and health professionals, where it was proven that there was a transmission of the virus from one person to another, and direct contact with symptomatic patients was the crucial risk for an extended transmission, and the permanent dissemination itself may depend on an unknown transmission of asymptomatic carriers (76). Another analysis in China reported that of the infected patients found, 3.5% corresponded to health professionals, 1.9% of the patients had a history of contact with wild animals, 31.3% had recently traveled to Wuhan city and 72.3% of non-residents in Wuhan had a connection with people from Wuhan (25, 76), which resulted in an increased concern regarding the ease of the virus in transmitting through several pathways (81).

Previously, it was generally thought that SARS-CoV-2 propagated only through respiratory droplets, but in more current studies it was found that its ability to be transmitted through other possible pathways (81). The transmission capacity that the SARS-CoV-2 has possibly is justified in part by its structure and also by its general tenacity, that is, its strong adhesion to surfaces or fluids. SARS-CoV-2 has in its structure, one of the most rigid external protective layers among all CoVs, which can result in the formation of more stable viral particles, causing greater resilience of them when they come into contact with bodily fluids or surfaces, remaining the virus viable for a certain time (81, 82).

Having said that, there are currently several modes of transmission of SARS-CoV-2 between humans (**Figure 4**), which can be categorized in detail as follows:

(I) **Respiratory droplets –** In this first mode, the SARS-CoV-2 is transmitted via respiratory droplets, in the case of a patient infected when coughing, sneezing, singing, breathing, and speaking (83). For this, the intervention of an access point is necessary, in this case, of mucous membranes (nose, mouth, and eyes), which allow these infected droplets (size > 5-10 μm in diameter) reach the mucous membranes of the other healthy person, this occurs when the infected person that presents respiratory symptoms of COVID-19 is distanced within 2 meters of the same healthy person (84). Thus, these small aerial droplets that include the virus can be inhaled by other susceptible persons who are within a radius of lower than 2 meters. The concern would be even greater if the droplets remain in the air and viable for some time (76, 81). Moreover, another means to take into account is related to the movement of dust through the air, since inhalation of fine particles

containing the virus can be directed to the innermost bronchial and alveolar zones of the lungs (85), since in a study it was observed in infected patients from three hospitals in China that the values of the highest positive rates for the presence of viral RNA were in the bronchoalveolar lavage fluid, sputum and nasal swabs (86). The contamination of spaces through the airflow can originate a viral transmission, via infectious droplets (81, 87).

(II)   **Direct contact –** In a study it was found that 71.8% of non-local residents of Hubei contracted COVID-19, having been in contact with people from Wuhan City and about 88.0% of health professionals with COVID-19 from China were in the Hubei region, according to data from 475 hospitals in 30 provinces in China (76).

(III)   **Indirect contact –** This type of transmission occurs when droplets that include SARS-CoV-2 land on smooth or rough surfaces (the virus being more stable on smooth surfaces) such as tables, door handles, telephones, glasses, plastic or stainless-steel surfaces (the virus is stable for 72 hours), among other inanimate objects that are capable of holding and transporting infectious organisms from one individual to another. The virus is transmitted from these same surfaces directly to the mucous membranes, or through the already contaminated fingers that touch in the mouth, nose, or eyes (76, 81).

In an initial analysis of patients identified with COVID-19, it was possible to identify the different transmissions to which they were subjected within a shopping center and in the results, it was detected that the spread of the virus was impossible to happen, only through the transmission of respiratory droplets, but it can also occur, by direct contact with other infected individuals, asymptomatic individuals or indirect contact with contaminated objects that were in space (81, 88).

Another example that confirmed this transmission was the registration by the Guangzhou Center for Disease Control (CDC) of a patient confirmed for COVID-19, whose surfaces of objects in his home were discovered SARS-CoV-2 (89). In a study on a cruise ship, more than 800 confirmed cases were identified, where viral RNA was noticed by some surfaces of objects inside the cabins of symptomatic and asymptomatic people, which potentiated the role of indirect contact transmission in objects contaminated with the virus and, moreover, of all samples collected from the surfaces, 80.4% tested positive for the presence of viral RNA, which sustains considerably the confirmation of this mode of transmission of the virus (81, 90).

In several studies it has been determined that the SARS-CoV-2 can live up to 5 days, under the conditions of a temperature of 20°C and humidity between 40.0 – 50.0%, however, in dry air can resist for less than 48 hours, decreasing its viability after 2 hours (76). It is important to note that from what is known, from surface samples of objects contaminated with SARS-CoV-2, it was not possible to isolate the virus in a viable way, which suggests the limitation of data regarding the transmission of the virus by this route (81).

(IV) **Asymptomatic transmission –** At least two cases of asymptomatic infections were reported, which demonstrated a contact history through a first pre-symptomatic patient who was later diagnosed with COVID-19, resulting later in the transmission of the virus to three other healthy family members (76). Another case was recorded in a study on 24th January 2020 by *The Lancet* magazine, where a family grouping was infected by SARS-CoV-2, since five of these relatives had a history of travel to Wuhan, having contracted COVID-19 and upon returning to Shenzhen city, the son was confirmed as asymptomatic for the disease, since he did not present any symptoms of fever, respiratory tract or diarrhea, but through radiography were detected pulmonary opacities in ground glass, a common condition in this type of disease (89). From these cases, several asymptomatic patients were identified by many chinese cities, in which most of them contained a relevant epidemiological history (89).

A study by *Nature Medicine* observed that asymptomatic infections, compared to symptomatic patients, may occur due to more debilitated immune responses and subclinical manifestations to a SARS-CoV-2 infection, since these, have lower levels of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies in the acute disease phase (period over which viral RNA can be identified in a respiratory sample), reduced levels of IgG antibodies and neutralizing antibodies, during the initial convalescence phase of the disease (8 weeks after hospital patients leave) and also low amounts of 18 pro- and anti-inflammatory cytokines (related to the immunological response) (89, 91). Another cause is related to the virus itself waiting for the appropriate time for its reproduction or replication and thus invading the host cells. One way to understand this mechanism is through investigation in asymptomatic patients, through blood tests that indicate signs of an immune response and may help in the diagnosis of asymptomatic or pre-symptomatic occurrences (89). In another analysis, it was also possible to

demonstrate the ability of asymptomatic patients to constitute a means of transmission of the virus, by the detection of a viral load in these patients similar to that of symptomatic patients (89). Therefore, before the appearance of symptoms, infected people may not be isolated, becoming in a possible and relevant mobile viral source, so this type of transmission has aggravated the complications to stop the spread of the disease, but on the other hand, the number of existing asymptomatic patients is reduced and, normally, are not responsible for large-scale SARS-CoV-2 transmissions (76, 89).

(V) **Interfamily transmission –** This mode of transmission inside each family gathering, which includes relatives and friends that subjected themselves to contact with infected patients is quite usual (25, 76, 89). In a study conducted in China, it was observed that 78.0% to 85.0% of occurrences in large group junctions arose because of interfamily transmission in cities such as Sichuan and Guangdong (76). Compared to data from studies on other respiratory viruses and their main routes of transmission among humans, it was demonstrated that in SARS-CoV, hospital transmission and transmission among family members followed in only 22.0% to 39.0% of cases and in MERS-CoV in 13.0% to 21.0% of cases, which relatively to SARS-CoV-2 are significantly below (89).

(VI) **Transmission via aerosols –** The confirmation of airborne transmission in a respiratory virus, as was the case with SARS-CoV, does not necessarily imply that this same transmission occurs in another, because each of them presents its virological characteristics, such as incubation times, among others (92). In the case of SARS-CoV-2, it uses the S protein present in its constitution, with a view to interact with a greater affinity relative to SARS-CoV with the ACE2 receptor, expressed considerably on the surface of type 2 alveolar epithelial cells in humans, since the alveolar epithelium constitutes the thinnest layer in the respiratory tract, resulting in lower protection and more direct access to ACE2 receptor, which favors in the virus infection process (25, 92). The airborne transmission occurs when large respiratory droplets evaporate or by dust particles, which contain the virus inside the droplet nuclei with diameters less than 5 μm, which leads to the virus keeping in the air for a longer time and moves by several meters away (81, 84). This type of transmission becomes a high risk when the process of aerosolization of particles occurs, especially in procedures performed in the treatment of this type of patients in more severe conditions, as is the case of

endotracheal intubation, bronchoscopy, and cardiopulmonary resuscitation (81, 84). Thus, in one study it was observed that SARS-CoV-2 could remain stable in the air for 3 hours, in artificially generated aerosols with a particle size < 5 μm, which may suggest a potential means of infection for the virus and its strong ability to survive in the air (92, 93). In closed spaces with little ventilation, aerosols can remain in the air between 24-48 hours, managing to disperse for several meters to tens of meters (76), which suggests that the virus can spread in non-ventilated environments and with high levels of viral aerosols (89).

Currently, the National Research Council has confirmed that existing investigations support the hypothesis that SARS-CoV-2 can be transmitted through bioaerosols, originated directly through the expiration of infected patients (93, 94). An example that demonstrates that the aerial transmission of SARS-CoV-2 may be a product of what happens in our daily lives is what happened in a choral group in Washington state, in which after 3 weeks of the event, of the 60 people present, 45 were infected and contracted the disease (93, 95). Therefore, the implementation of appropriate measures is essential in order to prevent the route of the aerial transmission of SARS-CoV-2 inside spaces with little or no ventilation, for example, the frequent circulation of fresh air and not recirculated air, makes it possible to effectively dilute the concentration levels of the virus in the space concerned (93, 96). As noted daily, the transmission of SARS-CoV-2 continues to vary between countries, regions, and even cities. In a study of identified cases conducted in different cities in China, it can be found that the variability of environmental factors, such as ambient temperature, relative humidity, and ozone concentration levels, in the period between January and March 2020, may have influenced the transmission potential of SARS-CoV-2 (93). These observations imply that the environmental conditions mentioned above are possible to adapt, with a view to reduce the transmission route of SARS-CoV-2, namely the use of ozone generators inside hospitals or other affected spaces, in order to inactivate SARS-CoV-2 and its contagion by air (93).

However, it is necessary to take into account the existence of other environmental factors and their influence on the survival of SARS-CoV-2, as they can vary depending on the space and require different adjustments, for this, it is necessary to discuss what could be more economically viable for the country concerned (93).

Finally, the number of viral particle samples that are viable by air is still very low, being needed still more investigations (76, 92).

(VII) **Ocular transmission –** The ability of the virus to be transmitted by the eye was recently examined by researchers and in a general context, in certain studies, the presence of viral RNA was found in conjunctival samples, while in others the evidence is still reduced relative to the detection of the virus in this type of sample (81, 97). In *The Lancet* magazine carried out a study, where was referred to doctors to take into account the transmission of SARS-CoV-2 via ocular surfaces since infected body droplets and fluids can perfectly contaminate the conjunctival epithelium present in human eyes (89). For this mode of transmission, there is a record of the case of a doctor, without any eye protection, who was infected during an investigation in Wuhan on January 22, 2020, presenting symptoms of conjunctivitis in the left lower eyelid area, 2 days before the start of COVID-19 (76, 89). Around this case, more studies occurred, where it was possible to detect the SARS-CoV-2 in tears and conjunctival secretions of this same patient who had COVID-19 (76).

In an analysis, published in *JAMA Ophthalmology*, 38 patients confirmed with COVID-19 from a hospital center in China it was possible to investigate which the types of ocular manifestations that were present in these patients, and it was concluded that approximately one third (31.6%) demonstrated consistent ocular anomalies, which usually occurred in patients with more severe symptoms of the disease, among the manifestations we have conjunctivitis, whose signs are conjunctival hyperemia, chemosis or increased secretions (81, 98).

A recent case was recorded relatively the first patient diagnosed with COVID-19 in Italy and this showed symptoms of bilateral conjunctivitis, in addition to fever, respiratory symptoms nausea, and vomiting. Thus, a way of showing that SARS-CoV-2 was present in the ocular samples, it was through its inoculation into Vero E6 cells, from one of these samples positive for viral RNA, observing a cytopathic effect caused by the virus after five days and a viral replication confirmed by the reverse transcription polymerase chain reaction (RT-PCR) technique, through the extraction of a sample of purified RNA in a cell growth environment. Consequently, all these results have meant that eye secretions in patients with COVID-19 may include SARS-CoV-2 in its constitution as an infectious agent and represent a possible source of infection (81, 99).

In contrast, other studies have shown limitations regarding ocular transmission. For example, a study was conducted in 17 patients with COVID-19, where tear samples were collected and all tested negative for the presence of SARS-CoV-2, although nasopharynx samples had positive results. Likewise, in patients who had upper respiratory tract infections, there was no evidence of viral shedding by tears, indicating that transmission of the virus through tears is possibly reduced (81, 100). Another example was a study that covered 67 suspected patients and others confirmed for COVID-19, 63 of which were laboratory confirmed. Of these 63 patients, one of the patients tested positive for conjunctival samples by PCR technique, while two other patients obtained possibly positive results, although in none of the three patients there were ocular symptoms (81, 101).

(VIII)   **Fecal-oral transmission –** When there is evidence of fecal transmission of SARS-CoV and MERS-CoV, including their survivability in patients' stool, possibly the SARS-CoV-2 can also be transmitted via this route and, moreover, in the case of ACE2 receptor and TMPRSS2 protease expression, these are also present on the surface of the cells of the intestinal and renal epithelium, which can allow SARS-CoV-2 to infect these tissues and penetrate stool, demonstrating its ability to spread via the fecal-oral route and lead to Infection by SARS-CoV-2 in the gastrointestinal tract (81, 89, 102, 103).

The first case of COVID-19 identified about this mode of transmission took place in the USA. Further studies made it possible to identify SARS-CoV-2 in stool and anal swabs of patients with COVID-19, moreover, 23.3% of patients continued to test positive in the stool, although viral RNA is no longer detectable in the respiratory tract of patients. Furthermore, the SARS-CoV-2 was identified in the gastric, duodenal, and rectal epithelium (76). As was the example of the case record of an asymptomatic patient for COVID-19, where the presence of the virus was detected in the stool for 42 days, although the nasopharynx sample having tested negative (81, 104).

Adult and pediatric patients after recovery of their own symptoms of pneumonia by COVID-19 were discharged from the hospital, since they met the necessary requirements, such as the results of nasopharynx tests being negative. However, it was possible to detect viral RNA in stool samples of these same patients for much longer than in the previous study, which implies that although these individuals regain their health status, continue to be able to transmit and disseminate the virus,

which makes the transmission of SARS-CoV-2 by fecal-oral route more alarming (81, 105, 106).

On the other hand, despite a more prolonged viral identification in the stool, it was important to discuss whether in fact, these viral particles could be infectious and whether or not they had the ability to propagate by fecal-oral route (81). Thus, in one study, the presence of the live virus in stool was described, is possible to cultivate the SARS-CoV-2 together with Vero cells (cell line isolated from renal epithelium cells extracted from the African green monkey), isolated from a stool sample of a patient who had severe pneumonia caused by COVID-19 (81). In another analysis published in *Journal of the American Medical Association (JAMA)*, it was found that this mode of transmission was also capable of causing wide contamination of the space around symptomatic patients with COVID-19 since we have as an example, the collection of samples from the room of a patient with fecal matter to test positive for the SARS-CoV-2, by the RT-PCR technique, before the usual cleaning occurs, which comprises the surface of the toilet, inside the toilet, on the door handle itself, among others, presenting all positive results. Soon after cleaning, these same samples tested negative, which suggests that the present measures applied for decontamination are efficient (81, 107).

Furthermore, it was discussed and analyzed the possible aseptic interventions that the transmission of SARS-CoV-2 by fecal-oral route can imply in the control of infections, particularly in areas with sanitation needs (81, 108). These new findings have resulted in more stringent care with regard to the handling of stool samples from patients with COVID-19, since in Australia, SARS-CoV-2 has already been detected in untreated wastewater and without sanitation conditions (81, 109). Consequently, in this same study, the need for warnings directed specifically at hospitals about the proper handling and disinfection of the sewers was also analyzed, since there is a gradual increase in concern about the appearance of this new means of transmission of the virus by the fecal-oral route (81, 108).

(IX) **Vertical transmission –** The SARS-CoV-2 to have a sequential nucleotide homology of 79.0% and 51.8%, compared to SARS-CoV and MERS-CoV, respectively, to suggest that the risk of vertical transmission of SARS-CoV-2 is similar to that of these mentioned viruses (89). One example was the case of a baby born at Wuhan Children's Hospital on 2nd February 2020, whose mother tested positive for COVID-19, later umbilical cord blood and placental tissue were

collected and analyzed, having a negative result for SARS-CoV-2, however the collection of nasopharyngeal swabs samples in the newborn after 36 hours of birth tested positive, but in this case, a transmission via respiratory droplets or direct or indirect contact, are also possibilities that cannot be ruled out (89).

In different studies, evidence of vertical transmission was demonstrated by the discovery in some newborns with mothers testing positive for COVID-19, of high amounts of IgM antibodies after birth (81, 110). In one of these studies, under the same conditions, the newborn contained, by analysis in a blood sample, a large amount of IgM antibodies 2 hours after birth, but nasopharynx samples obtained negative results for the presence of SARS-CoV-2, as well as maternal vaginal secretions (81, 111). Generally, the appearance of IgM antibodies only happens between 3-7 days after the infection, in part because of its molecular structure, since they have a very large macromolecular structure, it is not possible to transfer, through the placenta, from the mother to the fetus and the fact that it has verified a high amount of IgM antibodies and also abnormal results in a cytokine test of the newborn may imply that this has been infected in the uterus (81, 110, 111).

In another study, it verified in an analysis among mothers with COVID-19 with vaginal or cesarean deliveries (6 and 31 cases, respectively), that two of the newborns by cesarean section tested positive for SARS-CoV-2, through the RT-PCR real-time technique, however, three other newborns by cesarean section demonstrated large amounts of IgG and IgM for SARS-CoV-2, but tested negative for the RT-PCR test. In the case of the vaginal deliveries exist a high risk of ingestion or aspiration of the cervicovaginal secretions or also by contact with infected perineal tissue, however, as the data refer to the transmission of the virus by cesarean section, this risk is low or almost zero, which led to the conclusion that the transmission rate of SARS-CoV-2 by vertical route was reduced or null for deliveries by cesarean section, although vaginal deliveries did not have data available (81, 112).

A specific case of a newborn who was born by cesarean section at 32 weeks of gestation, without any indication of being infected by SARS-CoV-2, whose mother had COVID-19, was tested by RT-PCR technique a sample of the amniotic fluid and this tested positive, as well as a second test by nasal and oral route to the newborn, after 24 hours of birth. On the other hand, in samples of vaginal secretions, umbilical cord blood, and in the first nasal and oral test performed on

the newborn, its results were negative. Therefore, the samples that showed positive results, possibly indicated that the newborn was affected intrauterine by SARS-CoV-2 (81, 113). In contrast, in another study in nine patients with COVID-19 who were subjected to cesarean section deliveries, six were analyzed and tested the same type of samples, mentioned above, for SARS-CoV-2, further adding a sample of breast milk, and all obtained negative results (81, 114).

In another analysis, it was possible to verify the effects that SARS-CoV-2 infection in pregnant women can cause in newborns. This perinatal infection by SARS-CoV-2 can cause serious consequences in newborns, since some of them have experienced conditions of fetal distress, from premature deliveries, respiratory complications, thrombocytopenia, abnormal results of tests on liver function, and even the death itself (81, 115).

With the existence of several initial cases of mothers testing positive for SARS-CoV-2 or with COVID-19 (symptomatic), during pregnancy, the most indicated treatment to avoid infection of the virus in the newborn, consisted in the performance of delivery by cesarean section, after birth, isolating the newborn from the mother and applying a feeding with infant formula (116). The reasons why this treatment is implemented are related to previous knowledge, regarding other infections that occurred by SARS-CoV in 2002 and MERS-CoV in 2012 in pregnant women, which at the time resulted in some deaths, both of mothers and newborns (116, 117). Another reason may be the pandemic to have begun in China because rates of cesarean deliveries tend to be above 40.0%, since obstetricians, in problematic situations, recommend more the delivery by this route (116, 118). In this way, in a recent study, the known data on pregnant women with COVID-19 made it possible to critically analyze the risk of SARS-CoV-2 infection in newborns, through delivery mode (vaginal or cesarean section), infant feeding (breastfeeding or formula), and interaction between mother and baby (set or isolated) (116, 119, 120). However, although this study has been conducted a solid investigation of the literature, with the analysis of 655 cases of pregnant women and 666 newborns. The conclusions obtained from the results collected indicated that COVID-19 disease does not necessarily imply the execution of deliveries by cesarean section, a feeding with infant formula, or the isolation of the baby from the mother after birth (116). Consequently, cesarean sections should continue to be carried out, in normal obstetric conditions, and mothers who breastfeed and interact with their

babies, should continue to have the recommended hygiene care for SARS-CoV-2 infection, as is the case with the use of liquid-resistant surgical masks, if they are made available, at the time when feeding or treating the baby, since there is no evidence that the isolation of the mother's baby is advantageous. If all necessary care is taken, the temporary stimulation of the interaction between the baby and the mother will possibly facilitate the breastfeeding process and the bond between the two. The Isolation is advised only in case of need by medical recommendations (116).

Therefore, the transmission of SARS-CoV-2 to the neonatal by vertical route is still uncommon, rarely symptomatic and the virus infection rate does not present higher values whenever the newborn is born vaginal route, whether breastfed or is in contact with the mother, as mentioned earlier (116, 121).

Thus, the continuous increase in this type of cases implies that it is increasingly necessary in hospital reports and studies, the indication of what types of care is used, to list the geographical and hospital area of their cases and to collect samples from both mother and baby immediately after birth and register them in the medical literature, depending on the applied technique, in order to reduce the probability of the existence of overlapping or repeated cases and to present everything more detailed (116, 121).

In this mode of transmission, the study is still limited, due to a small number of samples, is needed even more research and sufficient evidence that supports the same, as the newborn samples analyzed from SARS-CoV-2 positive mothers, continue large part, until now, testing negative, since there are not enough or viable viral particles in the different constituents at the time of delivery conception (amniotic fluid, placenta, umbilical cord blood, breast milk, among others) or in the baby itself. Hence it is necessary more intensive research that allows confirming whether the SARS-CoV-2 can overcome the placental barrier (76, 81, 89, 121).

(X) **Sexual transmission –** Examples of studies that allowed to support this route of transmission was the case of a cross-sectional analysis carried out on 34 adult men in the recovery phase of COVID-19 disease and it was found that in none of the patients SARS-CoV-2 was identified in semen samples, after about 1 month of initial confirmation of the disease (81, 122). Nevertheless, these results do not completely rule out the hypothesis of being able to detect SARS-CoV-2 in the

seminal fluid of patients who are in a more acute phase of the disease (81, 122). Thus, in another study when examining 38 male patients confirmed with COVID-19, it was observed that six of the patients tested positive for the presence of SARS-CoV-2 in semen samples, of which four were at a more acute stage of SARS-CoV-2 infection, while the remaining two were in the recovery phase of the disease (81, 123). In the follow-up, a different study allowed the characterization of reduced gene expression of the ACE2 receptor overlap and TMPRSS2 protease on the surface of human testicles epithelial cells, which may indicate that SARS-CoV-2 possibly cannot infect testicular cells, preventing the viral entry through this mechanism. However, in another study, it was found that the ACE2 receptor is widely expressed in the testicles (81, 122, 124).

Furthermore, in another analysis, it was observed the absence of viral RNA in the vaginal environment of 35 female patients, which indicated still to have a lack of evidence to prove the sexual transmission of SARS-CoV-2 (76, 121).

In this type of studies, in which the possible routes of transmission of the virus are evaluated and discussed, there are certain limitations to be taken into account, among which there is the availability of research from studies already conducted, the sample size of patients involved in the study and the potential of the tests, compared with the need to provide detection methods (81). Besides, it is essential to highlight that although it has been possible to detect and quantify viral RNA in several samples of body fluids, on the other hand, the potential to extract samples of viable viruses and not from viral RNA fragments is extremely important in order to better understand the existing virus transmission routes and some of the related studies lack this type of data, it can be very difficult to reach plausible conclusions (81).

Therefore, since COVID-19 is still far from being fully understood is extremely crucial to carry out further investigations to be able to confirm and/or refute possible virus transmission routes, being this one of the main priorities (81, 121).
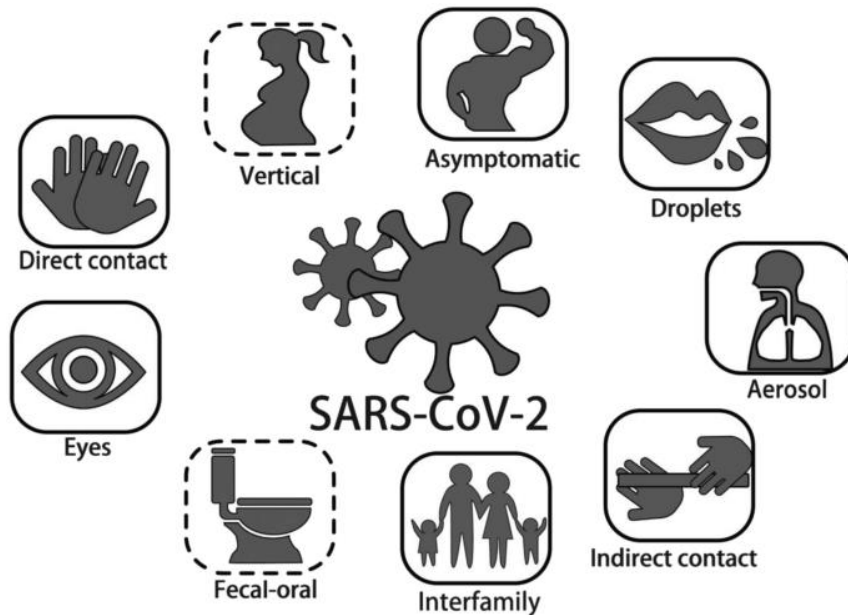
Figure 4 - Ways of transmission of SARS-CoV-2. The solid frames indicate confirmed modes of transmission whereas the dotted boxes have not yet been confirmed. Adapted from (76).

- The basic and effective reproduction number (R_0 and R_e)

This parameter is used to analyze the transmissibility of a virus and indicates the average number of new cases of infection produced directly by a single person already infected (125-127). In the case of index 0, this refers to the early stages of a pandemic, where the entire population of a region is susceptible to the virus, i.e. there is no immunity against the virus and also no countermeasure that can be applied to reduce transmission of the virus has been discussed or decided (125). Therefore, since geography and culture influence the countless people we come across in our daily lives, from touching to sharing food with them, it makes the R_0 rating differ between localities. Furthermore, R_0 is established in a context where there are no defined countermeasures, nor immunity against the new virus (125). Usually, instead, it is only possible to estimate the R_e, the effective number of reproduction, which has the same meaning as R_0, but in this case we are in the presence of countermeasures, and some people already immune against the new virus (125, 128). At the beginning of a pandemic, before the application of any countermeasure, its value is approximately equal to that of R_0, but after the implementation of control measures and prevention in reducing the transmission of the virus, the value of R_e is generally lower than that of R_0 (64, 125). When calculating R_0 of SARS-CoV-2, in several studies, the data indicated that each infected person directly originates, on average, between 2-4 more new cases of infected people (exceeding WHO assessments), that is, each infected patient can transmit the virus to two or three other healthy people, when we are in the absence of countermeasures, such as physical distancing (65, 69, 125, 129-131). In

one study it was possible to compare the R_0 values of COVID-19 with those of SARS (2.2-3.8) and MERS (2.7-3.9) and it was found that these were similar (69), but according to WHO estimates, at the beginning of the pandemic, the R_0 value of COVID-19 ranged from 2.0-2.5, however it is still debatable, being higher than SARS (1.7-1.9) and MERS (< 1), which indicates that SARS-CoV-2 has a greater potential to cause a pandemic (131). The R_0 value of SARS-CoV-2, being considerably higher than one, implies that SARS-CoV-2 has a high transmissibility capacity and thus a potential to cause outbreaks (64). However, several factors can influence the value of R_0, such as the evaluation period, the models applied and the data sets used, which must always be considered in any study estimate (65, 131). Then, after a person is infected, there is a time interval between exposure to the virus and the onset of infectiousness called the latent period, in which the virus is not transmitted, before the end of that period (125). Currently, the average latent period is evaluated at approximately 3 days, usually followed by an interval of time in which the infected person becomes infectious, that is, with the ability to transmit the virus, called the infectious period of about 4 days. However, the concrete durability of each of these periods differs among people since some can transmit the virus for a long time by presenting higher R_0 values (125).

In relation to R_0, it is known that the higher its value, the more intense the transmission capacity of the virus, and it becomes more complicated to control the pandemic (64, 74). Therefore, values of $R_0 > 1$ refer to an outbreak that will be self-sustaining, i.e. transmission between humans may continue (64, 65) if efficient control measures are not applied, on the contrary, $R_0 < 1$ values indicate that the number of new cases of infection will progressively decrease and thus the outbreak will eventually be stopped (64).

In fact, the meaning of these R_0 and R_e parameters allows informing whether the spread of a virus is occurring at a worrying rate, when there are not sufficient countermeasures adopted to the situation, so it is extremely important to mitigate the spread of the virus, through physical measures, such as the distance between people (125). The impact desired by physical distancing aims at R_e less than one, that is, to get each infected person currently to produce less than a new case of infection, which will ensure that the number of new infections eventually reduces (125). Thus, it is crucial to quickly achieve $R_e < 1$ values and maintain, since it is significantly more feasible than forcing R_e near zero values, through public health measures in each country and by the global union of them in combating this pandemic (64, 74, 125). As an example, we have the case of China, which is the first country affected by the pandemic, in March 2020 began to see more and more, a decrease in the number of new confirmed cases of infection. These data demonstrate that the application of control and prevention measures

was efficient, although globally, the situation remains highly serious. Hence it is necessary to maintain the measures implemented, in order to decrease the values of R_e (being in China, at an early stage of the pandemic, its value of 3.1) to optimal levels and thus control the possible transmissions of the new virus (64, 65, 130).

1.3.2. Risk Factors

SARS-CoV-2 infections have a higher incidence in adult male patients, with an average age ranging from 34 to 59 years (132, 133). In a study that evaluated patients confirmed for COVID-19, it was found that 50.5% demonstrated associated chronic comorbidities, from cardiovascular, cerebrovascular diseases, and diabetes, and in 23.2% of occurrences by COVID-19, there was at least one underlying comorbidity (134). The largest fraction of serious occurrences by COVID-19 consists of adults with ≥ 60 years of age and presenting associated comorbidities, since in those, SARS-CoV-2 has a higher probability of infection (5, 133, 135). However, these types of manifestations may also be related to coinfections of bacteria and fungi (5, 134). Of all the diseases mentioned, the most common is hypertension (14.9%), followed by diabetes mellitus (7.4%) (136). Similarly, in a report by the CDC of China, it also showed that hypertension was the health condition with the highest prevalence (12.8%), after diabetes mellitus (5.3%) and then cardiovascular diseases (4.2%) patients with COVID-19 (135, 137). Moreover, the seriousness of cases with COVID-19 was considerably related to the presence of other coexisting diseases (37.6%), compared to cases with non-severe COVID-19, i.e., without comorbidities associated with the patient (20.5%) (135, 136).

On the other hand, few occurrences of COVID-19 in children under the age of 15 have been recorded and, as an example, in a study published on 29th January 2020, conducted with 425 patients diagnosed with COVID-19 in the Wuhan region, it was found that there were no cases of children under 15 years of age (5, 132, 133, 138). However, that same month, 28 pediatric patients had been reported in China. As for clinical manifestations in infected pediatric patients, these differ according to the case, however, most of them demonstrated mild symptoms without fever or pneumonia, having a good prognosis (5, 139). In another analysis, it was possible to identify that although a child presented through radiological diagnosis, pulmonary opacities in frosted glass, this was considered an asymptomatic patient (5, 140). In short, children are less likely to be infected or, when infected, demonstrate lighter clinical characteristics compared to adults, therefore, it is likely that the parents of these children will not seek treatment for them, resulting in an undervaluation of the incidence of COVID-19 in this age group (5).

Many of the deaths linked to COVID-19 in hospitalized adult patients are also due to the ageing of people, because in older age groups the immune system is weaker and suppressed, allowing the virus a higher reproduction rate (135, 141). Other risk factors related to a high possibility of hospital death from COVID-19, refer specifically to the higher levels of fibrin D-dimer in the blood, above the values of 1 µg/mL and also a sequential organ failure assessment score higher, that is, a scale that allows predicting mortality, depending on the degree of dysfunction of at least six organ systems of the organism and the higher its value, the greater will be the probability of death of the patient (141).

In this sense, what is known to be the evolution of severe to critical or fatal cases of COVID-19 (patients in Intensive Care Units or ICUs), is that the associated risk factors include sex (male), advanced age ($\geq$ 60 years), certain symptoms (dyspnea, abdominal pain, anorexia, among others), primordial laboratory anomalies (lymphopenia, increase in the number of blood cells, among others), disorders in blood clotting (increased levels in the blood of fibrin D-dimer, in approximately 20.0% of cases), high sequential organ failure assessment scores and underlying comorbidities, such as hypertension, diabetes, severe pneumonia, cardiovascular diseases and cerebrovascular diseases, whose detection at an early stage of the disease, can help the doctors identify the patients with poor prognosis (3, 65, 130, 141). However, to define the risk factors for COVID-19, further studies are needed (135).

1.4. Pathogenesis

The process of pathogenesis of a virus begins with the interaction between the S protein of the virus and the different receptors presented by susceptible human cells. Then, by effectively entering the cells, the genetic material, namely the RNA of the virus that is released into the cytoplasm, begins to replicate and synthesize certain sequences that lead to the production of profitable ancillary proteins and facilitate the adaptation of the CoV to its human host (142). At the level of genome composition between CoVs, several modifications are regularly recorded that are the result of processes such as recombinations, exchanges, insertions or deletions of genes and which may be a justification for the occurrence of past outbreaks (142). Therefore, the classification of CoVs is constantly modified and according to the most up-to-date classification made available by ICTV, the CoVs are divided into four genera, mentioned above, which include a whole 38 unique species (142, 143).

Thus, several mechanisms can intervene in the process of pathogenesis of CoVs. As examples, SARS-CoV tends to connect to the ACE2 receptor, while MERS-CoV is more targeted to connect to the DPP4 receptor (142, 144). After the binding, a cascade of signals is activated,

where the genetic material of the virus is properly inserted into the cytoplasm of the target cell. This viral RNA responsible for regulating the expression of structural and non-structural polyproteins, undergoes a polyadenylation and is encapsulated. Proteins are cleft by specific proteases that demonstrate a similar activity to chymotrypsin (142, 144). Through replication and transcription, the formation of protein complex leads to production of RNA of negative sense or (-) RNA. Subsequently, these (-) full-length RNAs formed ultimately apply as models to process positive-sense RNA or (+) RNA (142, 145). So the total structural proteins of the virus are translated from a subset of 7-9 subgenomic RNAs, formed by a discontinuous transcription process. This protein complex already obtained is defined to include the viral genome, which results in the creation of a nucleocapsid during the process, which will be released to the lumen of the endoplasmic reticulum, in order to complete the intracellular cycle. The newly formed virions are later transported to the outside of the infected cell by exocytosis. After this moment, the new CoVs formed and released would already be able to infect a wide variety of human cells, which comprises lung, renal, hepatic, intestinal and lower respiratory tract cells, including T lymphocytes (142, 144).

- Incubation period

Another important parameter to be evaluated in patients infected by SARS-CoV-2 is their incubation period, which is described as the interval between the earliest possible date of contact, or exposure with a source of transmission (represented by a species of wild animal, or a suspected person, or confirmed for infection) and the earliest possible date for the onset of symptoms, that is, cough, fever, fatigue, myalgia, among others (136, 146). This parameter allows to notify about several fundamental activities for public health related to infectious diseases, from active monitoring, surveillance, control and modeling (146). Active monitoring requests that individuals possibly exposed contact local health authorities and report their health status in a daily basis. It is also necessary to understand the period of active monitoring, to minimize the risk of loss of infections by SARS-CoV-2 and thus, health departments effectively apply their limited resources (146).

From several estimates of analyses related to the mean incubation period for COVID-19 it was possible to determine that this is about 5 (range of 2-7) days, being similar to that of SARS, however can go up to 14 days (136, 138, 146-148). Estimates of the mean incubation period of SARS-CoV-2 are in accordance with those of other CoVs in humans already known, as the case of SARS with an average of 5 (range of 2-14) days (146, 149), MERS with an average of 5 to 7(range of 2-14) days (146, 150) and human CoVs not SARS with an average of 3 (range of 2-5) days (146, 151). Estimates of the mean interval from the beginning of symptoms to

admission to the hospital were 7 (range of 3-9) days (152). On these hospitalized patients, the average age differs between 47 and 73 years, corresponding in most studies to a higher prevalence of males, by about 60.0% (136, 153, 154). The hospitalized patients confirmed for COVID-19, between 74.0% and 86.0% are at least 50 years old (152, 154).

For the current period of active monitoring or quarantine period for infected patients, the recommended by the US CDC is 14 days (146, 155).

1.4.1. Pathogenesis in the Respiratory system

Of all organ cells that can be infected by SARS-CoV-2, those of the respiratory tract have a higher tendency, hence pneumonia is one of the first clinical features observed in patients diagnosed with COVID-19 (16, 67, 138, 142), but this is only one element of SARS, and may manifest in some cases.

The radiological features common in patients confirmed for COVID-19 comprise predominant bilateral infiltrates, in the lower lobe from the X-ray image of the thorax and present bilateral and peripheral ground-glass opacities in the lower lobe and / or consolidation on chest computed tomography (CT) (156). These chest CT shows irregularities, and particularly in patients with COVID-19, are defined by diffuse peripheral ground-glass opacities (**Figure 5**) (157). Ground-glass opacities have imprecise margins, air bronchograms, a smooth or uneven interlobular or septal thickening and thickening of the adjacent pleura (157). In one study it was possible to verify that in early stages of the disease, about 15.0% of patients with COVID-19 may have normal results on a CT scan of the chest and about 40.0% may have normal results on chest X-ray (136).

The rapid development of irregularities is possible during the first 2 weeks after the onset of symptoms, subsequently they will progressively reduce (157, 158).

However, the results of a chest CT in these patients are shown to be nonspecific, as they overlap with another type of infection. Therefore, the reliability of chest CT diagnosis in patients with COVID-19 is still limited. One example was observed in certain hospitalized patients, whose RT-PCR tests showed positive results, which confirmed SARS-CoV-2 infection, but CT imaging results were normal. On the other hand, in cases of abnormal chest CT imaging results, associated with COVID-19 patients, they were accomplished days earlier the identification of SARS-CoV-2 RNA in those patients (157, 158).

With the worsening of SARS, this can result in more severe and excessively complicated situations to control, such as septic shock, metabolic acidosis and coagulation dysfunction (142, 159). Regarding radiological research on COVID-19-associated pneumonia, it was found that

in approximately 1 week after the onset of signs and symptoms in patients with COVID-19, gradual lung lesions are commonly identified (142, 160). However, in the 2nd week the lesions worsen more and these result in the formation of irregular reticular opacities, along with ground glass opacities, being possible to identify by CT at the age of 4 weeks. Therefore, in one study it was possible to observe that 85.7% (54/63) of patients with pneumonia associated with COVID-19 demonstrated a higher evolution of the disease, caused by the high extent of ground glass opacities, which were followed early by CT (142, 161). In a specific patient who showed signs of recovery from the disease it was possible to identify pulmonary fibrous cords, since inflammatory secretions had been absorbed (142, 162). In a prolonged period of the disease, difficulties in patients with severe pneumonia associated with COVID-19 may include several fibrotic changes that are usually observed in the final stages of lung injury, such as reticulation, interlobular septum thickening, and traction bronchiectasis (142, 163).

In other viral respiratory diseases, such as influenza, at the immunological level, one of the possible conditions that occurs is an extensive lymphopenia and in patients with COVID-19 in early stages of the disease, this condition may also occur, whenever SARS-CoV-2 infects and eliminates T cells from lymphocytes. Besides, an inflammatory response triggered by a virus, includes an innate and adaptive immune response, from humoral immunity and cell-mediated. This viral inflammatory response compromises the lymphopoiesis process and, in turn, increases lymphocyte apoptosis (59, 164, 165).

For more advanced stages of SARS-CoV-2 infection, as the viral replication process intensifies, the entire epithelial-endothelial barrier of the pulmonary capillaries is affected. Not only the epithelial cells of the alveoli are infected by SARS-CoV-2, but also endothelial cells from pulmonary capillaries, allowing to increase the inflammatory response and induce an influx of monocytes and neutrophils at the site (166). The formation of interstitial mononuclear inflammatory infiltrates and edema appear as matte glass opacities in the CT. Subsequently, a pulmonary edema occupies the existing alveolar zones, through the formation of a hyaline membrane, as happens in acute respiratory distress syndrome in early stages of the disease (166). Another condition such as bradykinin-dependent pulmonary angioedema may also contribute to the development of the disease (167). Together all these conditions, from the disturbance of the endothelial barrier, to the alveolar-capillary dysfunctional transfer of oxygen, result in a potential compromised diffusion of oxygen, which are main characteristics of COVID-19 (168).

In severe or critical stages of COVID-19, an unexpected activation of the coagulation process is observed and, in turn, a reduction of the factors for this same process (169, 170). In a clinical

report from Wuhan city in China it was found that of 183 patients who died of COVID-19, 71.0% had diffuse intravascular coagulation (169). Thus, in the presence of this condition, lung tissues become inflamed and together with pulmonary endothelial cells, may lead to the formation of microthrombi and provide for a high incidence of thrombotic complications, such as deep vein thrombosis, pulmonary embolism and arterial thrombosis, which can evolve to limb ischemia, ischemic stroke and myocardial infarction, in extremely ill patients (168, 171). Furthermore, the emergence of viral sepsis, described as a life-threatening organ dysfunction caused by the deregulated response of the host concerned to SARS-CoV-2 infection, can further facilitate the process of failure in several organs of the patient (168, 172).
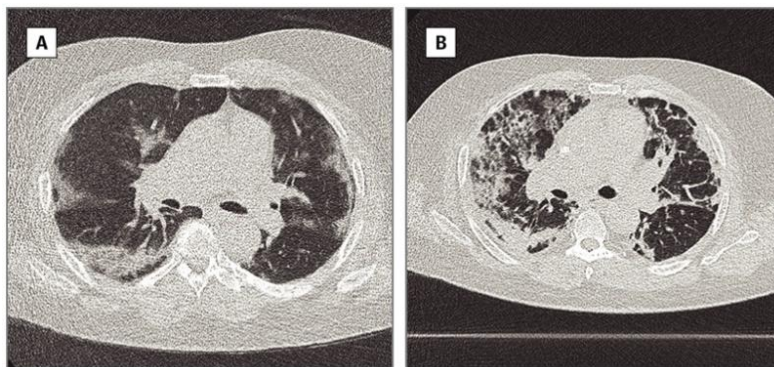

Figure 5 - (A) Transverse thin-section CT scan of a 76-years-old man, 5 days after symptom onset, showing subpleural ground-glass opacity and consolidation with subpleural sparing. (B) Transverse thin-section computed tomographic scan of a 76-years-old man, 21 days after symptom onset, showing bilateral and peripheral predominant consolidation, ground-glass with reticulation, and bronchodilatation. Adapted from (156).

1.5. Clinical characteristics

In patients with COVID-19 there are several clinical features that may vary depending on the stage of the disease in which the patient is. From a study conducted in China to 44,672 patients with COVID-19, it was observed that 81.0% of these patients demonstrated mild manifestations, 14.0% severe manifestations and, finally, 5.0% already critical manifestations, such as respiratory failure, septic shock and /or multiple organ dysfunction (137). In another analysis conducted in the United Kingdom of 20,133 patients hospitalized with COVID-19, it was found that 17.1% of patients had been admitted to ICUs (154).

However, through the analysis of several studies it was possible to verify that although approximately 25.0% of patients infected with SARS-CoV-2 present morbidities, between 60.0 - 90.0% of those who are hospitalized have associated comorbidities (137, 152-154, 173). Thus, these comorbidities associated with those that are most common in hospitalized patients

correspond to hypertension (between 48.0 - 57.0% of patients), diabetes (17.0 - 34.0%), cardiovascular diseases (21.0 - 28.0%), chronic lung disease (4.0 - 10.0%), chronic kidney disease (3.0 - 13.0%), malignancies (6.0 - 8.0%) and chronic liver disease (less than 5.0%) (152, 153, 173).

Based on the analysis of a set of studies, in patients hospitalized by COVID-19, the most frequent symptoms presented are fever (between 70.0 - 90.0% of patients), dry cough (60-86%), shortness of breath (53.0 - 80.0%), fatigue or tiredness (in 38.0% of patients), myalgia (15.0 - 44.0%), nausea/vomiting or diarrhea (15.0 - 39.0%), headache and weakness (25.0%) and, finally, rhinorrhea (7.0%) (136, 152-154, 173, 174). Atypical symptoms such as isolated gastrointestinal symptoms are also possible to be presented by this type of patients (174). Other dysfunctions such as olfactory and/or gustatory are also identified in 64.0 - 80.0% of these patients (175, 176). In extreme cases of anosmia or ageusia, these may represent the only symptoms in about 3.0% of patients (174, 176).

At the level of common complications in COVID-19, these include impaired functions of organs such as the heart, brain, lung, liver, kidneys and also the coagulation process itself. In the heart, COVID-19 is capable of causing various conditions, from cardiac injury to large amounts of troponin (7.0 - 17.0%), myocarditis, cardiomyopathy (acute heart failure), ventricular arrhythmias and hemodynamic instability (177, 178). For the brain, acute cerebrovascular disease (3.0%) and encephalitis occurs at a severe stage of the disease (up to 8.0% of patients) (179, 180). Other venous and arterial thromboembolic events occur between 10.0 - 25.0% of patients hospitalized with COVID-19 (181, 182). Patients with COVID-19 who are in the ICUs, possibly between 31.0 - 59.0% occur this type of thromboembolic events (171, 182). For lung, cases of pneumonia (75.0%) and acute respiratory distress syndrome (15.0%) are common in hospitalized patients and hypoxemic respiratory failure (between 17.0 - 35.0%) are usually treated in patients hospitalized with COVID-19 in the ICUs, between 29.0 - 91.0% of these patients, require invasive mechanical ventilation (154, 173, 183). In addition, these hospitalized patients may contract acute kidney damage (in 9.0%) and liver dysfunction (in 19.0%), due to high amounts of aspartate transaminase, alanine transaminase and bilirubin, and also in the coagulation process, present bleeding and coagulation dysfunction (between 10.0 - 25.0%) and septic shock (in 6.0%) (173, 174, 181, 183, 184).

In the case of rare complications in COVID-19, these are found in patients at a more critical stage of the disease that include a macrophage activation syndrome and cytokine storm, i.e., a secondary hemophagocytic lymphohistiocytosis (185).

For the younger age group, about 2.0 - 5.0% of patients with COVID-19 found by laboratory have ages below 18 years, with a median of 11 years. Children confirmed for COVID-19

present slight symptoms of the disease and are mostly restricted to the upper respiratory tract, and these are rarely hospitalized (186). It is not yet clear why children are not so susceptible to COVID-19. However, there are some likely reasons, which include that children have less strong immune responses, i.e., in the absence of a cytokine storm, they may also present partial immunity compared to other viral exposures and lower rates of contact with SARS-CoV-2 (186). Although most paediatric occurrences are mild, there is a small number of cases (less than 7.0%) of children hospitalized by COVID-19 who develop a severe disease, where it is necessary to apply mechanical ventilation (186). In recent times, a rare multisystemic inflammatory syndrome similar to Kawasaki's disease (187, 188) has been identified in children infected with SARS-CoV-2. This type of syndrome is rare in children (with a prevalence of 2 in 100,000 people aged < 21 years) (189).

In Table 1, it is possible to observe and compare the three main outbreaks of CoVs in the world (SARS-CoV-2, SARS-CoV and MERS-CoV), regarding some of their epidemiological and clinical characteristics.

1.6. Detection methods of SARS-CoV-2 infection

1.6.1. Conventional, swab-based molecular tests

The RT-PCR technique represents a nucleic acid amplification test, with the objective of identifying the RNA of SARS-CoV-2 in respiratory tract samples, such as nasopharyngeal or throat swabs. This is considered the conventional technique for the diagnosis of SARS-CoV-2 infection (86, 132, 190, 191). In this method, there is a diversity of targeted virus RNA genes used by different manufacturers, with much of the tests referred to one or more genes of proteins E, N, S, RdRp and ORF1 (190).

In most symptomatic patients for SARS-CoV-2 infection, viral RNA collected from nasopharyngeal swabs is evaluated by cycle threshold (Ct), and it is possible to identify viral RNA on the first day the symptoms appear and reach its maximum dose of alerting, passing the first week of the beginning of symptoms (190). This Ct parameter is defined as the number of appropriate amplification cycles, so that a fluorescent signal is produced. Thus, lower Ct values constitute higher viral RNA loads. Therefore, Ct values below 40 at the clinical level represent a positive PCR result for SARS-CoV-2 infection and Ct values > 40 represent a negative PCR result for SARS-CoV-2 infection (86, 190, 192). However, the diagnostic criterion recommended by the CDC of China is that for Ct values below 37 is a positive PCR result, these individuals are considered clinically suspicious and in the case of Ct values between 37-40 it is recommended to repeat the test (132, 192). Positive viral RNA by PCR

begins to reduce upon arrival of the third week and subsequently becomes undetectable (190). However, it was observed that patients hospitalized in severe or critical condition had lower Ct values, compared to Ct values in mild occurrences, which indicates that the positive PCR result may last for more than 3 weeks after the onset of symptoms from disease, when most mild occurrences continue to produce inconclusive results for PCR (190, 193). Nevertheless, a "positive" PCR result reflects only the identification of viral RNA in the samples collected but does not necessarily mention the presence of the virus in its viable form (190, 194).

However, the sensitivity in this type of test differs depending on the time at which it is performed, in relation to the time of exposure with the virus. From a model study, it was possible to determine a sensitivity of the test is 33.0% after 4 days of exposure to the virus, a value of 62.0% on the day of the onset of symptoms and a value of 80.0%, after 3 days of the onset of symptoms (86, 190, 195). The emergence of false-negative test results is provided by some factors, from the adjustment of the technique for sample collection, the period of exposure to the virus and the origin of the sample itself. Respiratory samples from the lower respiratory tract, for example bronchoalveolar lavage fluid, are associated with higher sensitivity than upper respiratory tract samples, such as nasopharynx smears (156). In a study that took 1070 samples from 205 patients confirmed for COVID-19 in China, it was observed that the highest positive rates of RT-PCR test results to SARS-CoV-2 RNA were in samples of bronchoalveolar washing fluid (with a value of 93.0%), followed by sputum samples (72.0%), nasal swabs (63.0%), and pharyngeal swabs (32.0%) (86, 190). The SARS-CoV-2 detection is also possible by stool, but it was not confirmed in urine (86). Another possible source of samples for the use of SARS-CoV-2 may be saliva, whose need for personal protective equipment is lower and a smaller number of swabs, since the sample collection can be done by the suspect patient himself (self-sampling and non-invasive) (196).

1.6.2. Saliva-based molecular tests

Non-invasive tests for SARS-CoV-2 are needed for dealing with collection in children and in order to alleviate requests of resources to accomplish testing (197).

Self-sampling saliva consist in an individually spit into a collecting equipment (198). In alternative of a nasopharyngeal swap can be a great improvement in preventing nosocomial infections by going to collect the specimen. Besides, being more comfortable, less invasive, this does not require a professional (197, 198).

Some studies demonstrated that viral loads of this new CoV in saliva are higher during the first five days, which can lead to less false negatives results and increase the sensitivity (197,

198). On the other hand, there are other ones that showed that saliva sample produce antagonist results, because different samples from the same person have had different outcomes. This shows a low reliability on this specimen and more studies and needed to accomplish the results pretended (198).

1.6.3. Methods based on isothermal amplification

This methodology allows to amplify a target sequence of DNA or RNA, in a simpler and exponential way, to its identification, and compared to the PCR technique, it does not require thermal cycles. These detection methods include isothermal processes that allow the connection of a primer and subsequent amplification using a polymerase, whose function allows the displacement of the chain, dividing the hybridized chain of the target sequence to be identified. The resulting amplified gene products are possible to be identified through photometry (199).

From the high diversity of detection methods developed, there are two in particular applied to SARS-CoV-2. The first called loop-mediated isothermal amplification (LAMP), consists of a simple tube that allows DNA amplification, however, by combining reverse transcriptase and LAMP (RT-LAMP), it is possible to identify RNA (199, 200). RT-LAMP showed efficient identification of SARS-CoV-2 in clinical samples of patients with COVID-19, using several loop primers specific to the orf1ab and S genes, which allowed the amplification of the target sequences, obtaining sensitivity and specificity values of 100.0% and an average diagnosis time of < 30 min (201). The second isothermal method consists of an amplification of nucleic acids, through a reverse transcriptase and a recombinase polymerase (RT-RPA detection test) that allows the binding of the primer, specific to the N protein gene of SARS-CoV-2, in the homologous sequence of the target double stranded DNA. After amplification by the extension of the primer interceded by polymerase, sensitivity and specificity values of 100.0% were reached in this diagnostic test (202, 203).

1.6.4. Tests based on Cas13a

These tests combined the CRISPR technique (Clustered regularly interspaced short palindromic repeats) which has been used in DNA or RNA identification when pre-amplifying nucleic acids, along with the enzymological component of CRISPR Cas that specifically recognizes DNA or RNA sequences (199, 204).
The Cas13a enzyme is an RNA trans-endonuclease use on CRISPR technique in the identification of SARS-CoV-2. One of the main peculiarities of this technique is the use of the

enzyme Cas13a which identifies and binds to specific sequences of the virus RNA. This activates the enzyme and then the remaining sequence is not specifically cleaved, called "collateral" cleavage, in order to amplify a signal and identify the nucleic acid. In addition, the technique with Cas13a can be associated with the RT-RPA technique, which allows the amplification of target nucleic acid, in order to obtain results with greater sensitivity, being designated the sherlock technique (specific high-sensitivity enzymatic reporter unlocking), which also allows the application of reading techniques, such as fluorescence, colorimetry, lateral flow, among others, in order to quickly identify a wide range of targets (199, 204, 205). Cas13a is directed to the S protein and orf1ab genes of RNA present in SARS-CoV-2. The binding to the target zone leads to the activation of the enzyme that will cleavage the reporter probes, causing an increase in fluorescence output signals and proving the presence of viral RNA (199, 204, 205).

1.6.5. Serological testing

Another way to be able to identify an infection by SARS-CoV-2 indirectly is by evaluating the immune response of the host, in relation to the same infection, by identifying antigen-specific antibodies present in SARS-CoV-2 (targeting mainly N proteins and RBD of S protein) in the collection of serum samples (190, 192, 206). This type of serological diagnosis is particularly essential in patients in mild or moderate disease, and may manifest later, going beyond the first two weeks of the onset of the disease. Furthermore, this diagnosis is becoming a crucial tool in order to understand the extent of COVID-19 in society and thus be able to detect people who are immune and possibly protected to be infected by other one (190).

The biomarker with greater sensitivity and earlier use in serological testing correspond to total antibodies, which begins to increase during the second week of the beginning of symptoms (190, 207). Although in the enzyme-linked immunosorbent assay test (ELISA) for the antibodies IgG and IgM be considered positive result, shortly after the fourth day the onset of symptoms. The highest levels of these antibodies happen between 2-3 weeks of the disease (190).

The performance of several serological tests for the presence of SARS-CoV-2 can aid in the diagnosis and allow an evaluation of immunological responses in relation to new vaccines (190, 208, 209). However, the detection of antibodies in this test may not provide immunity against SARS-CoV-2, because all antibodies produced in response to virus infection may not be neutralizing. In a second SARS-CoV-2 infection, it is not yet known how often they happen. It is also not known, if in the presence of antibodies, they can modify the sensitivity in a

subsequent infection or what is the period of durability of the protection of these antibodies (156). The measurement of IgM antibodies occurs during the first 5 days of infection, higher amounts of IgM are observed during the second to third week of the disease, while IgG antibodies are initially detected, about 14 days after the onset of symptoms of the disease (190, 208). In severe diseases higher titers of antibodies are identified (209). These serological assays are available covering point-of-care assays and also high-performance enzyme immunoassays. However, the efficacy, accuracy and validity of these tests are variable (210).

1.6.6. Antigen tests

In several research laboratories, from enzyme immunosorbent enzyme assay (EIA) platforms, as is the case of the ELISA technique, have developed lateral flow immunoassay test (LFIA) in order to quickly and qualitatively identify the SARS-CoV and SAR-CoV-2. This test consists of a portable strip that allows the measuring of antibodies or antigens of SARS-CoV-2 for a simple diagnosis, targeting the domains of subunits S1 and S2 in protein S or the N proteins present in SARS-CoV-2 (199, 211). The LFIA test consists of a strip that includes fixed test reagents contained in a cassette. On the strip, the test reagents constitute a layer of purified monoclonal antibodies (mAb) or recombinant antigens located in particular areas of a nitrocellulose membrane (199, 211). Then, when placing in the strip blood drops of a suspicious patient, the target of the mAb are the viral antigens, while the recombinant antigens are identified by the antibodies present in the blood sample of the infected patient. In addition, the strip also includes marked antibodies capable of connecting to the antigen concerned (199, 211). Thus, a positive result implies the binding of the recombinant antigen on the strip with the patient's antibodies, and also the binding of the marked antibody, which results in a colored signal. In a positive antigen result, it implies the connection of an mAb with the antigen of the patient (199, 211).

In relation to antigen-rapid identification kits, they typically have suboptimal sensitivity and specificity values (212, 213). However, for the development of more sensitive test kits it is possible to resort to two approaches based on specific and conserved domains of proteins present in SARS-CoV-2. A first approach is an initial treatment to concentrate the target antigen, and a second approach where mAb are applied to various epitopes of the antigen concerned (212). In two studies, a sensitivity interval between 93.0 - 100.0% and 100.0% specificity was observed for SARS-CoV-2 antigen tests by N protein-specific immunochromatography (212, 214, 215).

1.6.7. Serum virus neutralization assay (SVN)

SVN represents a serological test that assesses the potentiality of a patient's neutralizing antibodies against the infectivity of SARS-CoV-2 and thus minimize infection. For an analysis of these protective antibodies, this assay is recognized as being the most reliable for this purpose and may indicate the use of convalescent plasma as a passive treatment of antibodies in the fight against SARS-CoV-2 infection, especially in patients in severe condition of the disease (199, 216). Initial studies have indicated that a convalescent plasma transfusion is capable of preventing the viral replication process of SARS-CoV-2, allowing to protect a subject from an infection (217, 218). In regular diagnosis, this type of assay is not applied, but is the first to be used to combat SARS-CoV-2 virulence (199).

1.6.8. Next generation sequencing (NGS)

The NGS technique allows the total sequencing of about 30,000 nucleotides of the SARS-CoV-2 genome, constituting a method of SARS-CoV-2, through environmental monitoring and surveillance tests, at the same time also allows to notify about the origin of a strain and viral evolution. The respective new sequence is inserted into the biggest SARS-CoV-2 database called GISAID, where it is already possible to find more than 17,000 SARS-CoV-2 sequences from around the world (199, 219).

In this technique, viral RNA is extracted from clinical samples of patients in the same way as in the RT-PCR test, and then purified in order to remove cytoplasmic and ribosomal RNA from humans (199, 219).

In **Table I** it is possible to observe in a summarized way some methodological characteristics in relation to some detection tests to SARS-CoV-2.

Table 1 - Summary of principal SARS-CoV-2 detection methods highlighting the patient sample required for testing, material being tested, and key features. Adapted from (199).

Method	Sample	Detected material	Key features
RT-PCR	• Nasopharyngeal swab • Oropharyngeal swab • Bronchoalveolar lavage • Tracheal aspirates • Saliva	Viral RNA	• Duration: 2–5 hours • Accuracy: High • Primary use: Gold standard diagnostic test • Cost: High (Reagents and Equipment) • Major limitations: Time and cross reactivity with other viruses (false positives)
EIA	• Blood • Nasopharyngeal swab	Antibodies/Antigens	• Duration: Hours • Accuracy: High • Primary use: Screening for exposure • Cost: High (Reagents and Equipment) • Major limitations: Cost, antigen detection is less accurate than RT-PCR
LFIA	Blood (finger stick) Saliva	Antibodies/Antigens	• Duration: Minutes • Accuracy: Lower than RT-PCR and EIA • Primary use: Rapid screening • Cost: Low • Major limitations: Lower accuracy particularly in antigen testing
SVN	Blood	Antibodies	• Duration: 5 days • Accuracy: High • Primary use: Detect neutralizing antibodies (convalescent plasma) • Cost: High • Major limitations: Duration
Emerging Methods			
Isothermal amplification • RT-LAMP • RT-RPA	Blood (finger stick)	Viral RNA	• Duration: Minutes (<30 min) • Accuracy: To be determined • Primary use: Rapid screening • Cost: Medium (Specific reagents) • Major limitations: Requires validation
CRISPR/Cas13a	Blood (finger stick)	Viral RNA	• Duration: Minutes • Accuracy: To be determined • Use: Rapid diagnosis • Cost: Low • Major limitations: Requires validation
NGS	Blood (finger stick)	Viral RNA	• Duration: Hours–days • Accuracy: High • Primary use: Genomic profiling of virus • Cost: High (Reagents and Equipment) • Major limitations: Cost, mainly used for genetic mapping rather than diagnostic

Legend: CRISPR – clustered regularly interspaced short palindromic repeats, EIA – enzyme immunoassay, LFIA – lateral flow immunoassay, NGS – next generation sequence, RPA – recombinase polymerase amplification, RT-LAMP – reverse transcriptase loop-mediated isothermal amplification, RT-PCR – reverse transcriptase polymerase chain reaction, SVNA – serum virus neutralization assay.

1.6.9. SARS-CoV-2 in Exhaled Breath Condensate (EBC)

The use of swabs to collect samples from suspected patients is known as a very uncomfortable method. Having said this, it would be advantageous to find a quick and non-invasive option, and this is where breathomics would be important (220).

The concept of exhaled breathing involves a gas phase and another liquid phase. The gaseous phase comprises gases such as N_2, CO_2 and volatile organic compounds (VOCs), in concentrations in the order of picomolar. These VOCs can occur from cellular metabolic

activity *in vivo* and possibly by induction in pathological processes (220). On the contrary, the liquid phase includes exhaled breath condensate (EBC) and exhaled breath aerosols (EBA), which comprise an extensive variety of non-volatile molecules, from cytokines, chemokines, hydrogen peroxide, ammonia, adenosine, leukotrienes, isoprostanes, nitrogen oxide, peptides, DNA and RNA (221, 222). The evaluation has revealed to be a more current and non-invasive technique that allows the identification of biomarkers, resulting especially from the lower respiratory tract (223). The collect is done during the usual breathing, through the cooling and condensation of the exhaled breath itself. The identification of inflammatory markers corresponding to chronic diseases of the airways, such as cystic fibrosis, asthma and chronic obstructive pulmonary disease are already detailed in the literature (223, 224). In addition, the detection of compounds or biomarkers in exuded air in areas such as metabolomics, proteomics and genomics to identify respiratory and systemic diseases early, lately it has gained a high relevance. The EBC is not only made up of water, but also exhaled drops, which include imprisoned semi-volatile and non-volatile compounds, such as proteins, metabolites, small polar compounds, portions cellular, fatty acids, cytokines, bacteria and viruses. The droplets or aerosols result not only from the rupture of the surfactant in the lower airways, but also by turbulence in the upper airways (222, 223, 225). In the case of droplets, they are released at the time of expiration by coughing and sneezing, and their sampling and analysis are possible individually (220).

In this way, EBC, EBA and VOCs can play a crucial role in the diagnosis of COVID-19, since viral particles are transmitted through respiratory droplets, hence the EBC and the EBA are considered in the diagnosis (226). In this non-volatile part that constitutes exhaled breath, it was possible to identify RNA and viral DNA, as is the case of rhinovirus and influenza (227). Furthermore, in one study, sensitivity and specificity values of 66.0% and 100.0%, respectively, in the case of non-herpes virus identification, were observed in relation to an evaluation of bronchoalveolar washes (228).

Finally, this analysis of the EBC allows to provide a new research domain aimed at non-invasive tests. At this time, the existing diagnostic tests still demonstrate values of specificity and sensitivity far from perfection, and improvements are always needed until they reach the values that are intended (220). Therefore, investigations into volatile and non-volatile compounds present in exhaled breath would be extremely important for non-invasive and extensive screening of SARS-CoV-2, although it is difficult to achieve high sensitivity and specificity values, in the clinical validation of breathing devices (220).

2. Discussion: SARS-CoV-2 detection tests

There are several parameters that evaluate the detection tests for SARS-CoV-2 infection, including the method of sampling, the durability of the test, the associated costs, the sensitivity and specificity that allow to choose and apply the most advantageous test, depending on the situation presented. Thus, each detection test has its benefits and drawbacks.

Regarding viral RNA testing, the first to consider is RT-PCR, which in addition of being the most widely used detection test for SARS-CoV-2, one of its main advantages is the ability to analyze different types of samples (from nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage, tracheal aspirates, saliva, among others) and in large numbers in a period of 24 hours, demonstrating sensitivity values for the test of approximately 95.0%, which leads to be able to consider this test as a good test and very sensitive (229). Another advantage is related to the minimum limit for the identification of SARS-CoV-2 in RT-PCR, which is estimated at less than 10 copies per reaction, allowing early identification of the virus at low concentrations (230). As for the durability of the RT-PCR test, this varies between 2-5 hours (199, 211), depending on the kit used, which compared to the other detection tests to SARS-CoV-2, most of them have short-term duration. Nonetheless, although the RT-PCR test can be considered as a rapid test, more test kits are being developed that allow an even faster analysis. In RT-PCR test, the amplification of a specific gene in SARS-CoV-2 implies a positive result for the presence of viral RNA and this result must always be linked to clinical observations, history and epidemiological information of the patient concerned (199), in order to be able to draw conclusions regarding its final diagnosis. However, in relation to the disadvantages in the RT-PCR technique, developed kits can be complicated to execute, have high prices and the delivery of results is more time-consuming (192, 199). Additionally, in certain studies there were high rates of false-negatives in the diagnosis by RT-PCR of infection by SARS-CoV-2 (67, 134, 231). These incorrect results by RT-PCR are possible to be caused by different phases of the methodology, from sampling, storage, transfer, purification and inappropriate processing of samples. In swags, the quality of the extracted RNA also influences the final results (192). Then, there are other factors that can lead to false-negative results in RT-PCR test, such as the degradation of purified RNA, the existence of inhibitors in RT-PCR, and/or genomic mutations in the primer and probe region in the genome of SARS-CoV-2. On the contrary, the occurrence of cross-contamination between samples or cross-reactivity with other viruses during sampling, pipetting and processing or technical misunderstandings, may contribute to false positive results in the samples (192, 199). Even though there is a probability of the occurrence of these incorrect results, molecular diagnosis by this technique is nowadays

the most accurately and sensitively accessible solution for early and large-scale identification of SARS-CoV-2 (192).

In the case of serological or antibody tests, the simplicity of the method in the identification of antibodies against SARS-CoV-2 is the main advantage and are suitable for comparing several samples from a single patient. The ELISA technique, which is the most applied in these tests, presents positive rates of 85.4% in the identification of IgG against SARS-CoV-2 in patients and IgM between 75.6-93.1%, where sensitivity and specificity values for serum IgM of 48.1% and 100.0%, respectively, were observed. While in the serum IgG were 88.9% and 90.9%, respectively (208, 232). The highest values for sensitivity in these serological tests are identified 2 weeks after the onset of symptoms of the disease. The identification of IgM is less sensitive in relation to IgG, due to possibly the immunological response of IgM happening first, and there is later a decrease, which leads to a less strong signal of detection, whereas in IgG the signs are possible to be detectable more quickly and for a period of 20 days, after 2 weeks of the onset of symptoms (199). One of the solutions is the addition of a specific marker for these Igs, thus increasing the sensitivity value in these rapid serological tests or antibodies specific to respiratory viruses (233). Obtaining the results in these tests allows you to notify about the state of the infection in which the patient is and to identify previous exposure to SARS-CoV-2. Finally, this antibody test is also applied for the detection of Igs to SARS-CoV-2 in recovered patients and collect serum or plasma samples from them and thus, serve as human donors in the treatment of critically ill patients (218).

On the other hand, the results in serological tests for SARS-CoV-2 may differ depending on the period of the disease after the onset of symptoms and, in turn, the reliability of these diagnostic tests. Therefore, there are some concerns that are not yet clarified, as when the IgM or IgG specific to SARS-CoV-2 will allow their detection during the course of the infection, how long they persist after infection and the extent of protection of these neutralizing antibodies against a subsequent infection of the virus (199).

The parameters of sensitivity and specificity, in a generalized way, refer to the probability of occurrence of false negatives and false positives in the methodology of the tests. Therefore, given that the risk of resurgence of a SARS-CoV-2 infection is not yet known for COVID-19, the identification of one or two IgM and/or IgG antibodies in serological tests does not forcibly ensure immunity to a possible reinfection (199). Regarding the false negative results in this test, by definition, they do not exclude a SARS-CoV-2 infection, especially in suspected individuals who have been exposed to the virus. As for the false positive results in these serological tests, they are possibly caused by a previous or current infection of SARS-CoV (208) and probably non-SARS-CoV strains (199, 234). Therefore, it will be essential to conduct

a strict analysis in the diagnostic tests for antibodies in order to calculate the accuracy and reliability of the results of serological tests (199).

The LFIA diagnostic test has as its main advantage its feasible use, since only two drops of blood from the patient are required for sampling in the identification of SARS-CoV-2 and its antibodies. This test is very fast, since the result is obtained in about 15 min, through visual identification, in relation to RT-PCR test (2-5 hours). An identification of antibodies demonstrates prior exposure to the virus, while the identification of antigens refers to the active carriers of SARS-CoV-2. The sensitivity and specificity values in this test are comparable to other antibody and antigen identification tests, such as the ELISA technique (199, 211, 212). Regarding the disadvantages in these tests, the identification of SARS-CoV-2 in patients through the detection of viral antigens is more difficult to develop compared to the identification tests of neutralizing antibodies against SARS-CoV-2, as the formation of purified mAb against these target antigens is required. Furthermore, it is always necessary to evaluate and optimize these tests by collecting blood samples from infected patients (199, 211, 212).

The SVN test is an extremely solid and reproducible test that is used in the identification of neutralizing antibodies against SARS-CoV-2 in convalescent plasma samples from recovered patients, in order to detect the best candidates to use this treatment. Thus, when receiving convalescent's plasma samples, there are several parameters that can be monitored, from the viral load, the concentration of antibodies in the patient and, in turn, the immunological response of neutralizing antibodies by defining algorithms that allow to ascertain the factors between patient and giver that translate better clinical efficacy (199). This process requires living strain of SARS-CoV-2 and its availability is a process that involves a lot of regulation, limiting the development of SVN tests in the laboratory. Despite being a relatively inexpensive test, it is done manually, shows a long-term duration (5 days) and always calls for a very strict internal standardization and quality control (199).

NGS is very useful to compare in parallel several genomic sequences of different strains of SARS-CoV-2 from the GISAID database and allows to identify some new strain, depending on the emergence of new mutations and insert this in that database. Although this methodology is one of the broadest used in the design of SARS-CoV-2, the cost is relatively expensive and predisposes several steps in the preparation of the sample, not being applied in large-scale tests (199).

In addition, two other major disadvantages in this set of tests for screening for a SARS-CoV-2 infection refer to the way in which samples are taken, as it requires, in most cases, the presence of the suspected person (likely to be infected) and also consist of invasive methods (excluding saliva test), which sometimes cause discomfort in that same person.

3. Future Perspectives

In the last 10 months we have been assisting to a world record speed in the study of a new epidemiologic disease agent, the SARS-CoV-2 virus. Despite all the knowledge we been bringing up, we still are very far from being able to deal with the health care burden of this new arising infection, in an efficient way.

Although there are several tests that detect SARS-CoV-2 already validated and whose protocols are constantly optimized in order to reach ideal values in the parameters that evaluate them (sensitivity, specificity, among others).

The race to make a vaccine that is safe and secure is astonishing, because many companies are trying to achieve a correct and prolonged immune response combined with a maximum reduction in side effects caused by the same, through different formulations. Although the emerging of a vaccine is necessary, right now a quick and cheap diagnostic mean is what we need, so we can easily know if someone is infected and isolate them, and this way prevent the dissemination of the virus.

Currently, a vaccine is not available in humans against SARS-CoV-2, but there are about 320 possible candidates who are in the development phase (235). Thus, the methodologies applied include the use of nucleic acids (DNA or RNA), also the attenuation of inactive or living viruses, through viral vectors and, finally, the use of recombinant proteins or viral particles (236, 237). In the process of running for an efficient vaccine, there are some difficulties that include technical obstacles, such as understanding whether the S protein or RBD proteins of a virus are able to induce antibodies with greater protection, when the human body is exposed previously to an adenovirus serotype 5 (known to compromise the immunogenicity of the vaccine that uses a viral vector). An adjuvant is needed to help improve this condition in the vaccine (156). Other difficulties are related to the feasibility of producing and regulating the vaccine on a large scale, in particular providing safety and efficacy for human health and also legal obstacles, from the dislocation of technology and licensing pacts (156). In the case of SARS-CoV-2, S protein, present in its constitution, appears to be a potential immunogenic target for protection, however, it is still not certain whether the S protein in its total length or only RBD is satisfactory to prevent transmission of the virus (237). Other issues related to the possible duration of vaccine immunity are extremely important, since it is necessary to know the number of appropriate doses of the vaccine, capable of granting immunity against the virus (190, 237). Therefore, at this time there are at least thirty candidate vaccines to combat SARS-CoV-2 that are being tested in phase 1-3 clinical trials (235).

For future months, other new virus prevention approaches may appear, such as monoclonal antibodies, hyperimmune globulins, and convalescent titer (238). The strategy of being efficient may apply to high-risk people, such as health professionals, important workers in other areas and older adults, particularly the elderly who are in nursing homes or long-term care facilities (156).

Therefore, while an efficient vaccine or treatment against SARS-CoV-2 is not available, the need for constant non-pharmaceutical interventions worldwide is essential to fight this virus (239).

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