Covid-19 diagnosis: from advanced molecular tools and to the last frontier of artificial intelligence

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Note: highlighted in italic are information for non-biologists

Introduction

Since the emergence of the pandemic, the view of Covid-19 and of SARS-CoV-2 itself has consistently changed. The clinical characteristics of Covid-19 span from no symptoms at all, to mild symptoms, till organ failure, due to an exacerbated immune response (figure 1).

Figure 1

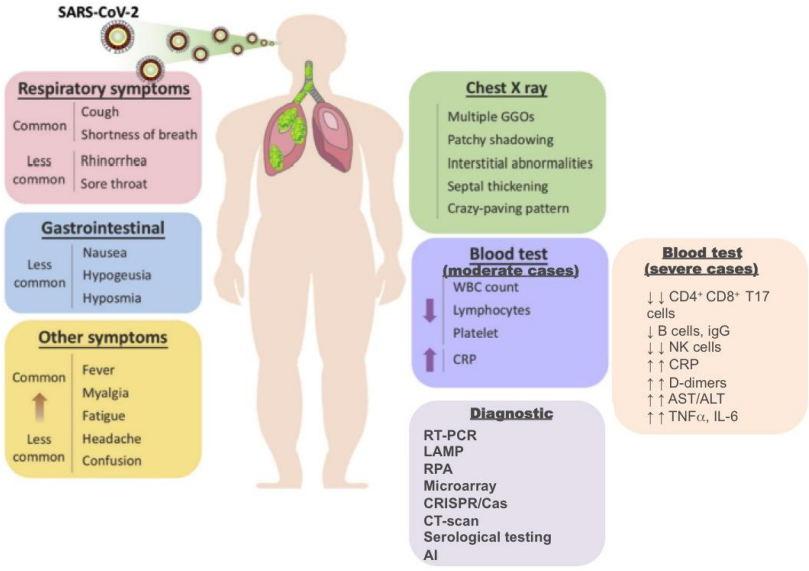

Figure 1. Overview of Covid-19 symptoms. Abbreviations: CRP= C reactive protein; AST=aspartate amino transferase; ALT=alanine aminotransferase; TNF-α=tumour necorsis factor α; IL-6=interleukin-6; RT-PCR=reverse transcription-polymerase chain reaction; LAMP=Loop-mediated isothermal amplification; RPA=recombinase polymerase amplification; CT=Chest Tomography; AI=artificial intelligence. *(Adapted from Tu et, al Int J Mol Sci, 2020*)

This variety of clinical manifestations probably depends on the viral load a patient has been exposed, on pre-and co-existing pathologies, age, gender and even genetics of the immune system (in particular of the HLA system). Indeed, it has been demonstrated that in severe cases the mean viral load is 60 times higher than in mild cases[1]. CoV-2 may infect

multiple tissues and may be recovered from different biological fluids, which represent the starting materials for Covid-19 diagnosis.

Covid-19: ARDS or MICROCLOTS?

The clinical course of Covid-19 has a broad spectrum of severity. As of february 2020, Covid-19 symptoms included fever (98%), cough (76%), dyspnea (55%), myalgia or fatigue (44%), sputum production (28%), pharyngodynia (12%) headache (8%), hemoptysis (5%), and diarrhea (3%)[2,3]. However, in mild cases, fever occurred only in 11% of patients, whereas the most frequent symptoms were pharyngodynia, cough, accompanied by hyposmia, nasal congestion and rhinorrhea[4]. Severe disease course often presents the characteristics of Acute Respiratory Distress Syndrome (ARDS), with dyspnea occurring 8 days after the onset of symptoms and rapid progression to septic shock and multiple organ dysfunction[2]. Requirement of intensive care unit (ICU) was necessary from 3% to 29% of patients. Complications were represented by RNAemia (*that is, detection of viral RNA in blood*), acute cardiac injury, acute kidney injury, secondary infections. Major co-morbidities were hypertension, diabetes and cardiovascular disease (CVD), at different percentage according to the considered published study. Chest computerized tomography (CT)-imaging of moderate-severe cases showed bilateral alterations in almost 90% of cases[3]. Laboratory parameters included the upregulation of several cytokines, lymphopenia and eosinopenia (see also Episode 4 for details). Despite these evidences, the rapid worsening of Covid-19 patients clinical conditions, within hours, has led to the hypothesis that the pathophysiology of Covid-19 was not “ARDS-typical”. Indeed, most of the patients showed preserved lung mechanics with relatively high respiratory compliance (that is: the lung gas volume is well preserved) in contrast with severe hypoxemia (*low oxygen concentration in blood)*. An explanation may be that, in these patients, lung perfusion is compromised, due to inflammatory-dependent microvascular pulmonary thromobosis. Indeed, the hyperactivation of the host immune system, characterizing severe Covid-19 cases, may locally activate the complement cascade, which not only directly damages the alveolar endothelium, but, recruiting leukocytes, boosts the inflammatory response, leading to the production of a plethora of cytokines (figure 1). Lymphocytes, macrophages, neutrophils also exert their pro-inflammatory function, leading to alveolar epithelial and endothelial massive damage and pulmonary microvascular thrombosis, as suggested by elevated levels of lactate dehydrogenase and D-dimer (figure 2)[5,6]. This local condition may potentially expand to the microvascular tree of other vital districts, such as kidney and brain. Based on these

evidences, it has been recently suggested that the lung damage in Covid-19 patient is mainly of vascular origin and MICROCLOTS (Microvascular Covid-19 Lung vessels

Figure 2

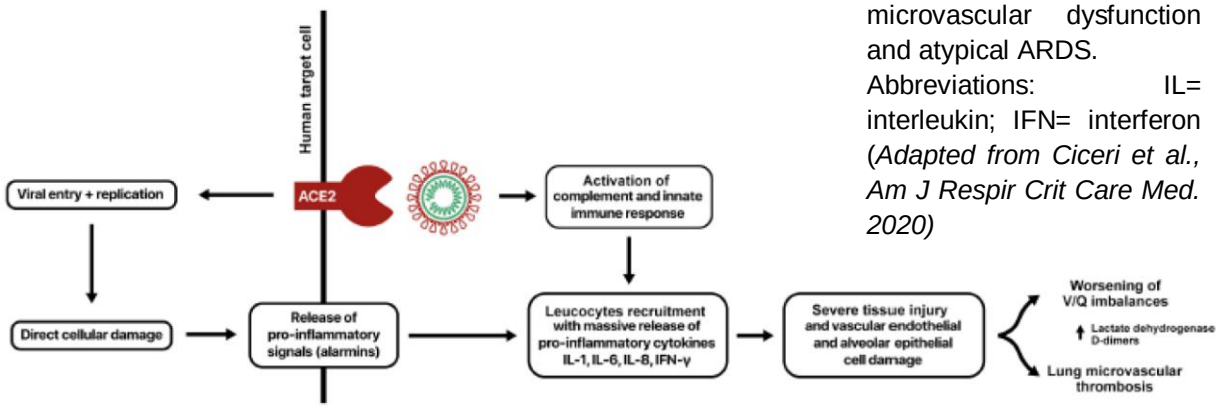

Figure 2. Cascade of events leading to pulmonary microvascular dysfunction and atypical ARDS. Abbreviations: IL= interleukin; IFN= interferon (*Adapted from Ciceri et al., Am J Respir Crit Care Med. 2020)*

Obstructive Thromboinflammatory Syndrome has been proposed as an alternate and novel definition of this atypical SARS-CoV-2 (CoV-2)-dependent ARDS[6].

Target tissues and transmission of CoV-2

- <u>Tissues.</u> ACE2-expressing tissues and cells are the direct target of CoV-2 infection (see also Episode 3). These include, not only epithelial cells of the upper and lower respiratory tract, but also vascular cells (such as pericytes[7]), oral buccal and gingiva, epithelial cells of tongue and oral

Figure 3

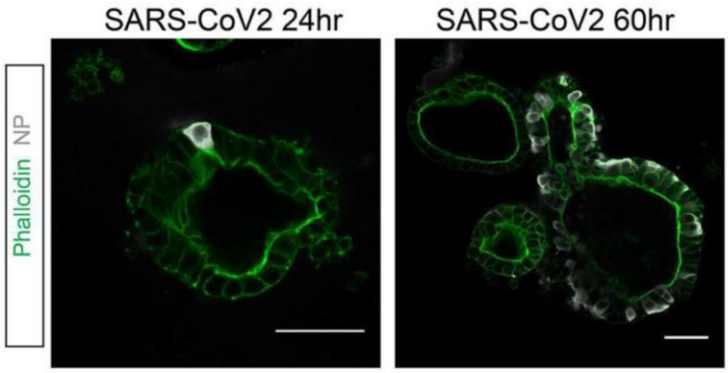

Figure 3. Immunofluorescence* showing the presence of CoV-2 nucleocapsid protein (NP, in white) on human intestinal organoids[§]. Phalloidin (in green) stains cytoskeletal actin. **Immunofluorescence is a technique which allows to visualize the localization of a protein on cells and tissues by recognition of specific antibodies bound to fuolrophores, enabling protein detection at a fluorescence microscope. [§]Organoids represent the most recent tools for "in vivo" studies. Basically, they reproduce a miniaturized version of the organ under investigation, starting from 3D cultures usually of tissue-specific stem cells onto extracellular matrices. (Adapted from Lamers et a., Science, 2020)*

mucosa[8]. It has been detected also in cells from salivary glands[9]. It has been demonstrated that epithelial cells of minor salivary glands ducts of rhesus macaques may be targeted by SARS-CoV after 48 hours from infection[10]. Therefore, it s conceivable that CoV-2 may also infect salivary gland ductal cells. Moreover, ACE 2 is also expressed in esophagus and other epithelial cells of the gastrointestinal tract, including enterocytes. Indeed, it has been recently shown that these latters express high levels of ACE2 and may be productively infected by CoV-2 (figure 3)[11].

- Transmission. As every respiratory virus, CoV-2 transmission relies on the emission of droplets, mainly by coughing and/or sneezing (figure 4a). Whereas an individual may emit by coughing about 75000 droplets/coughs[12], it has been recently demonstrated that ordinary speaking may produce a higher number of droplets, which may represent vehicles of infection. Furthermore, it has been shown that this number differs according to the voice loudness. Droplets size may differs from 1 to 500 μm, but, as small droplets evaporate quickly and larger droplets are subjected to gravitational settling velocities, droplets of medium diameter (that is from 30 to 50 μM) reach the maximum horizontal distance, which has been estimated in less than 1 m when breathing till 6 m when sneezing[13]. Loud speaking

Figure 4

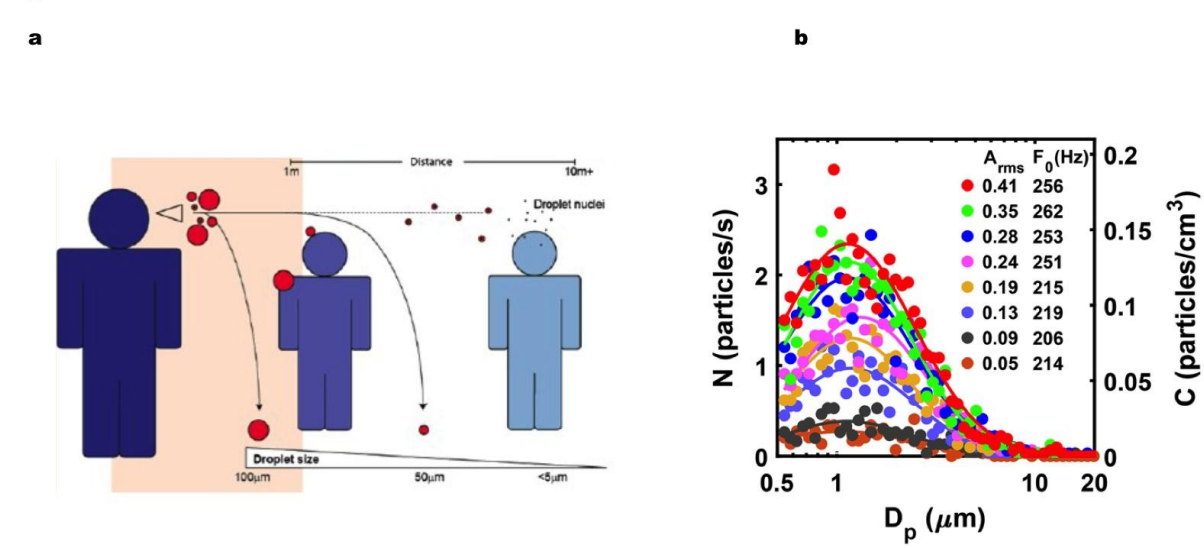

Figure 4. a) Relationship between droplets size and airborne dispersion. b) Size distribution of droplets while saying a letter, at 8 amplitudes, for 6 times at each amplitude. Abbreviations: A_{rms}=root mean square amplitude; F_0= fundamental frequency, that is the frequency at which vocal chords vibrate in voiced sounds. (*Adapted from International Federation of Infection control, www.theIFC.org, 2016; Asadi et al., Sci Rep 2020)*

may produce 2600 particles per second. These particles dehydrated quickly (resulting in droplet nuclei). For example, droplets with a diameter of 21-12 μM, with a volume of 60 to 320 nL, dehydrated rapidly to 4 μM. The half life of these particles is 8 minutes and, considering for CoV-2 a viral load in oral fluid of about 7 x 10^6 copies per milliliter (ml) (the maximum average load has been estimated in 2.35 x 10^{11}copies/ml[14]), it has been calculated that 1 minute of loud speaking may generate 1000-virion containing droplet nuclei, remaining in the air between 8 and 14 minutes. The probability that small particles contain virions declined from 37% of 50 μM diameter droplets, to 0.37% for particles of 10 μM, while the probability for these latter to contain more than one virion is negligible[15]. Smaller particles may remain indefinitely in the air; however, for dehydrated particles of 1 μM (starting from 3 μM hydrated droplets) the probability to contain a virion is 0.01%[15]. Although particle emission rate increase with loudness, the size distribution of particles is not affected

Figure 5

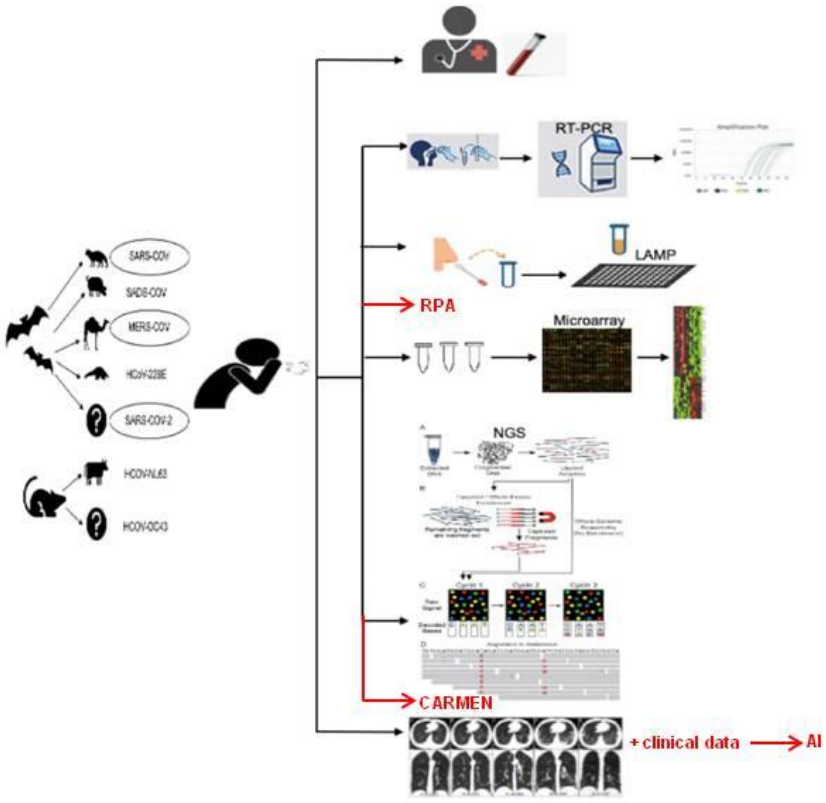

Figure 5. Overview of diagnostic tools used for CoV-2 detection. In red are highlighted the most recent advances.(*Adapted from Ozma et al., Infez Med, 2020)*

(figure 4b). Moreover, the existence of droplets "superemitters" has been demonstrated, which may explain the occurrence of CoV-2 "superspreaders"[16]. Fecal transmission has been also hypothesized and is still a debating issue. CoV-2 RNA copies have been detected in stool, independently from positive or negative naso-pharyngeal swab and from the presence or absence of symptoms[17]. The detection of viral RNA in stool does not mean that stool contain infectious virions. Nevertheless, it has been reported the presence of vital virions in stool specimens, as observed by electron microscopy[18]. The viral load of stool specimens has been determined recently[19]. 0.6-0.7/ml RNA copies of has been detected in stool specimens of patients without or with diarrhea, respectively. This is an important point, since CoV-2 may have low infective dose. Therefore, although it is believed that stool viral load should be low, fecal transmission should be considered as a concern. Furthermore, viral RNA has been detected also in urine and blood[18]. Contamination may occur also by contact with surfaces onto which droplets deposit. In fact, droplets half-life changes according to different materials[20].

Covid-19 diagnosis

In figure 5 is shown a cartoon depicting a variety of methods, currently employed to detect CoV-2 and allowing Covid-19 diagnosis.

- Molecular tools

a) Polymerase Chain Reaction (PCR)-based methods. The initial molecular identification of CoV-2 relied on reverse transcription-polymerase chain reaction (RT-PCR) methods from naso-pharyngeal or oro-pharyngeal swabs. *RT-PCR is a method based on the production and detection of multiple copies of a gene of interest - in this case, CoV-2-specific genes, such as the Nucleocapsid (N) gene – starting from a RNA molecule. The reverse transcriptase enzyme, using as a template total RNA molecules exctracted from cells, little sequences (oligonucleotides) as primers and nucleotides as "building blocks", synthesizes a single first strand of complimentary DNA (cDNA). Afterwards, special, heat-resistant bacterial DNA polymerases (such as the Taq polymerase, from Thermus Acquaticus or Pfu from Pyroccocus Furiosus), make multiple copies of the gene starting from the cDNA and specific oligonucleotides, recognizing the target gene, as primers (figure 6a). DNA products may be visualized on an agarose gel, which separate DNA molecules according to their molecular weight. However, this is a*

qualitative analysis. To monitor the quantity of a transcript in a given sample, real time-PCR has to be used. Real time-PCR methods are based on the emission of fluorescence as the DNA molecules increase in the sample. Double-stranded DNA binding dyes, like SYBRGreen, (less specific) or fluorescent reporter probes, like Taqman probes, (more specific) may be used. They differ as double-stranded DNA binding dyes bind to every double strand DNA molecule (figure 6b), increasing fluorescence as the amount of DNA molecules increases, while fluorescent reporter probes detect specifically those DNA molecules complimentary to the probe. In this case, fluorescence is released at the end of the DNA synthesis, when the reporter probe is degraded (figure 6c). In each case, the fluorescence is detected by a real time-PCR machine and the quantity of DNA molecules calculated according to the method described in Livak and Schmittgen 2001[21]. Specifically, at the beginning of the pandemic, the Food and Drug Administration (FDA) approved a SARS-CoV-2 commercial test system from Roche (cobas® SARS-CoV-2). This test takes 3.5

Figure 6

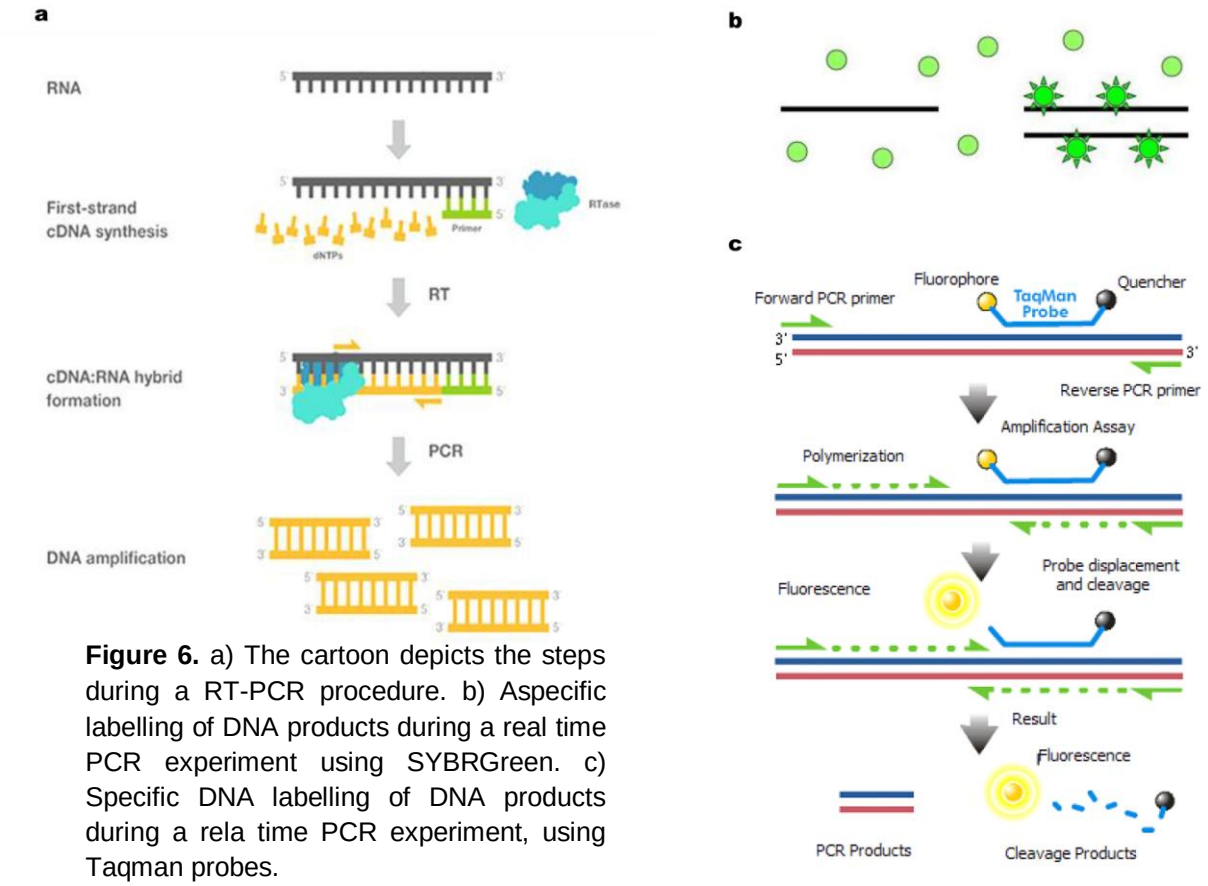

Figure 6. a) The cartoon depicts the steps during a RT-PCR procedure. b) Aspecific labelling of DNA products during a real time PCR experiment using SYBRGreen. c) Specific DNA labelling of DNA products during a rela time PCR experiment, using Taqman probes.

hours to yield the results. Therefore, later, another faster system has been

approved, the Xpert® Xpress SARS-CoV-2 from Cepheid Inc (USA), which yields the results within 45 minutes.

b) Loop-mediated isothermal amplification (LAMP) is a cheaper and time-saving method. Furthermore, by using multiple primers, it is more sensitive and specific. Detection of the final products may be performed by gel electrophoresis and/or real time-PCR. This method was already used for detection of other Coronavirus (SARS-CoV[22], MERS-CoV[23], HCoV-NL63[24]). With this method as few as 3.4 copies of MERS-CoV have been detected[23] and 0.01 plaque forming units (PFU) of SARS-CoV[22]. For SARS-CoV-2, LAMP has a detection limit of 2 x 10^2 copies per reaction[25].

c) Recombinase Polymerase Amplification (RPA). An evolution of PCR-based

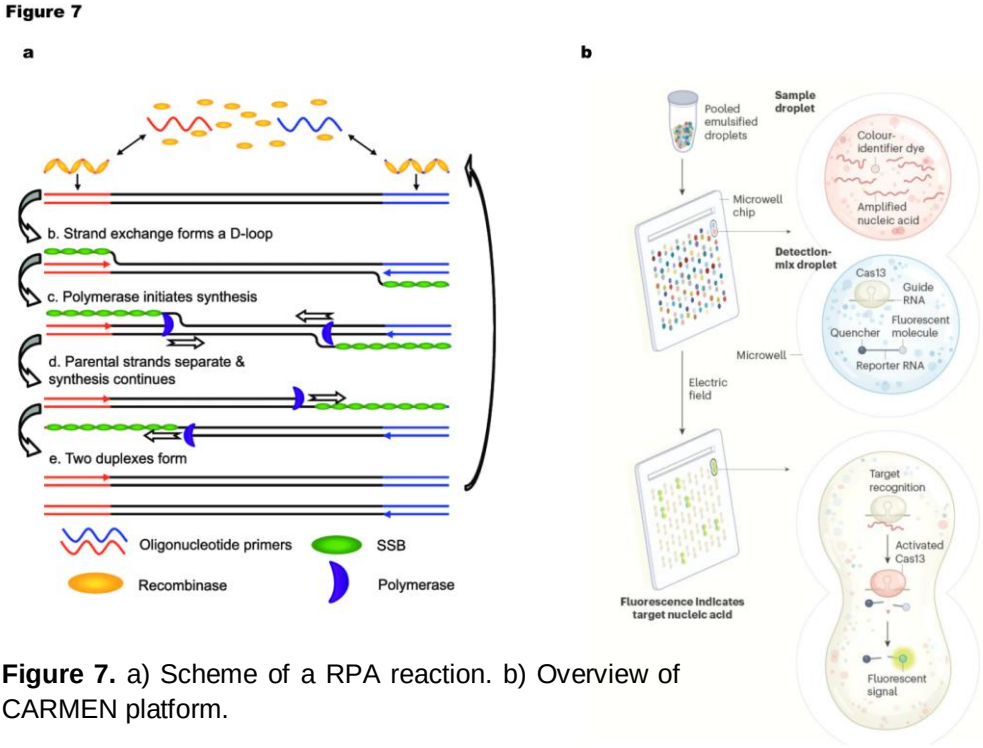

Figure 7. a) Scheme of a RPA reaction. b) Overview of CARMEN platform.

methods is RPA, which uses two opposing primers, as PCR, complimentary to the target sequence and complexed with the Recombinase protein, forming a D-loop in the double stranded target sequence. Primers are then extended by a mesophilic (*that is, stable at moderate temperatures*) DNA polymerase (figure 7a). If fluorescent probes are added, RPA may be monitored in real time. This method has been recently demonstrated to detect CoV-2 RNA within 20 minutes, being the first results available after 7 minutes[26].

d) Microarray-based studies may be also implemented for CoV-2 detection. Other Coronaviruses have been already detected by this method[27]. Basically, the viral

RNA is retrotranscribed in cDNA and categorized by specific probes. Labelled cDNAs are hybridized onto a microarray containing the probes, followed by a series of washes to remove unbound cDNA.

e) Next Generation Sequencing (NGS)-based methods. NGS methods may simultaneously detect multiple viral genes, by sequencing the pool of RNAs of infected cells. The bioinformatic analysis of the obtained sequences, starting from the alignment with other available sequences in GenBank, leads to the categorization of novel viruses. These methods are also used to identify viral genomic diversity and phylogenesis (see also Episode 2). For example, very recently, the analysis of 7666 genomic sequences of CoV-2 worldwide led to the identification of recurrent mutations, occurring independently overtime (homoplasies), in specific CoV-2 genomic regions[28].

f) CRISPR/Cas-based technology. The most recent advance for molecular CoV-2 diagnosis is the combinatorial arrayed reactions for multiplexed evaluation of nucleic acid (CARMEN), based on the CRISPR/Cas technology[29]. *The CRISPR/Cas system depends on the use of a RNA guide complexed with the Cas enzyme. If the RNA guide binds to a complimentary sequence in a target nucleic acid, Cas cuts the target.* CARMEN takes advantage of Cas13, which cuts only RNA and not DNA. In this method, Cas13 cuts reporter RNA in a non specific manner when is activated by the recognition of a specific sequence. PCR or RPA are needed in the first step to amplify viral nucleic acids (whether present). A fluorescent dye is mixed to give to the amplified RNA a specific colour code, in a ratio providing 1 to 1050 colour combinations. Different one-nanolitre oil emulsified droplets are then generated for all the different amplification reactions. Another series of emulsified droplets with unique colour codes are also generated. These coloured droplets contain a quenched fluorescently labelled reporter RNA and Cas13 bound to a guide RNA needed to detect a viral target. The mixed droplets (in a single tube) are loaded onto a chip containing microwells, capable to contain only two droplets. On the chip, each amplified nucleic-acid target is likely exposed to each detection mix, in multiple replicates in different locations. The exposure to an electric field leads to the merge of droplets pairs, initiating the detection reaction. If Cas13, in complex with a guide RNA, recognizes an amplified viral sequence in the same well, Cas13 is activated and generates a fluorescent signal from the reporter RNA, due to its nonspecific RNA-cleavage activity (figure 7b). This platform is

extremely innovative and permit the detection of more than a virus per experiment. It has been demonstrated to distinguish SARS-CoV-2 from other human coronaviruses, SARS-CoV and MERS-CoV[29].

Radioimaging

Chest computerized tomography scan (CT) is currently used to diagnose SARS-CoV-2 infection and Covid-19. The main radiological findings are the following: ground glass opacity (GGO, that is areas of opacity where the lung structures, including vessels, are still visible), consolidation (that is, area in which air in alveoli is substituted by fluid and/or cells), bronchial wall thickening and peripheral distribution. Less frequently, reticulation, crazy paving pattern (due to interlobular and intralobular septal thickening, intralobular interstitial thickening or to deposition of material in the air-occupied spaces at the borders of acini), intrathoracic lymph node enlargement and subpleural bands were observed[30,31]. CT-imaging may help in the diagnosis of false-negative patients, due to technical errors or for the presence of virions in the bronchoalveolar lavage fluid (BALF) and not in the upper respiratory tract.

Serological tests

Detection of antibodies is another method to indirectly detect CoV-2 infected individuals. Detection of nucleic acids may be efficient only in the acute phases of the disease, although some patients result positive even when

Figure 8

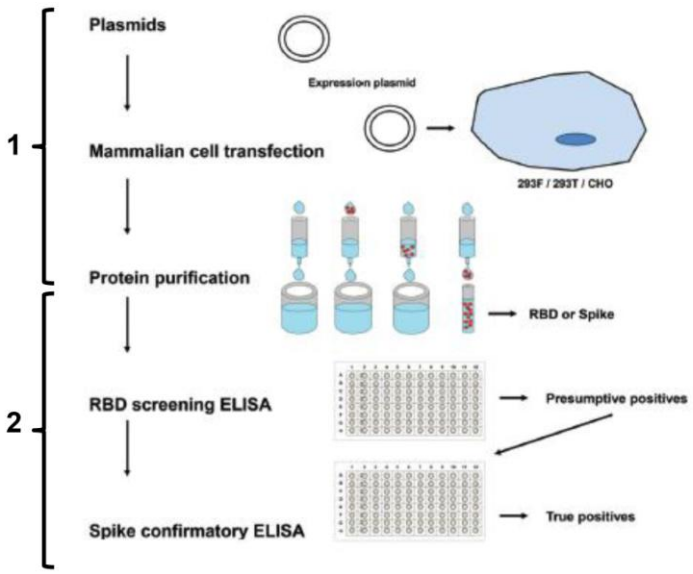

Figure 8. Entire procedure for the set up of a CoV-2 specific ELISA test. First, HEK293T cells are transfected with plasmids* encoding His-tag CoV-2 Spike RBD or and trimeric Spike. Therafter, proteins are purified from cells by Ni-NTA[§] purification system and immobilized on an ELISA plates. Patient sera are tested by loading samples into plate microwells and detection by a secondary antibody conjugated with HRP. Positive samples are detected by reading the plate in an ELISA reader at 490nm, after stopping the reaction with HCl.
**A plasmid is a circular DNA molecule used to express a variety of genes in both prokaryotic and eukaryotic cells.* [§]*The Ni-NTA system exploits the affinity of nickel nitrilotriacetic acid (Ni-NTA) for histidine (His).* Abbreviations: HEK293T=human embryonic kidney 293T cells, *where T means large T antigen from Simian Virus 40(SV40)*. HRP=horse radish preoxidase. *(Adapted from Stadblauer et al., Curr Protoc Microbiol, 2020)*

recovered since several weeks. Antibodies detection is also useful to identify those people potentially immune to CoV-2 re-infection. CoV-2 serological tests are based on the enzyme-linked immunosorbent assay (ELISA), *whose principle is the recognition of a specific protein (or part of a protein) by antibodies present in the serum of an individual.* The most specific are two-stage ELISA, in which a serum is first screened for the receptor binding domain (RBD) of Spike (S) and then for trimeric S (figure 8)[32].

Artificial Intelligence (AI)

RT-PCR-based CoV-2 detection, which is still routinely used to diagnose CoV-2 infected individuals, may be time consuming and may require multiple testing to ensure exclusion of false negatives. CT scan may not identify Covid-19 patients, as mild clinical manifestations may not be characterized by typical radiological Covid-19 signs (see above).

Figure 9

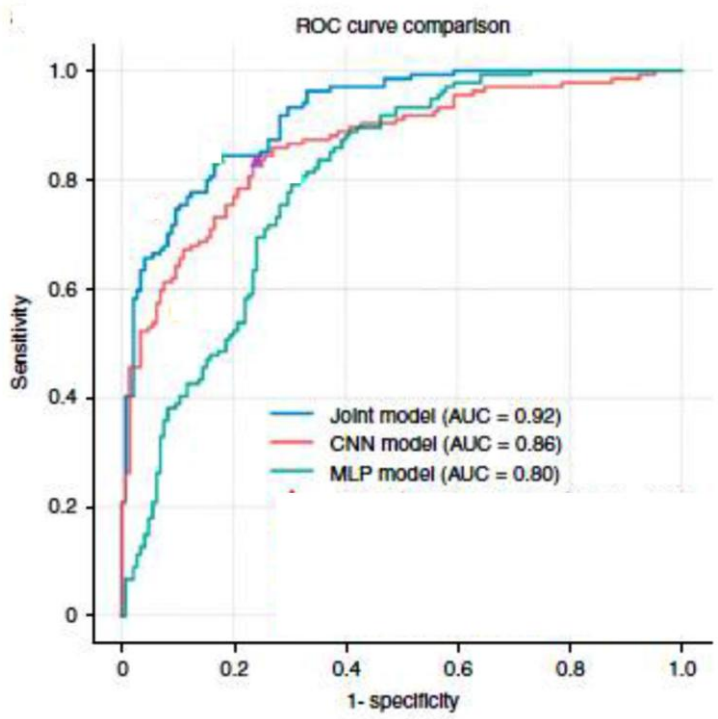

Figure 9. The plot illustrates the diagnostic ability (defined by Receiver Operator Curve, ROC) of the joint model (in blue), the model considering only the CT-scans (in red) and the model considering only clinical data (in green). Abbreviations: CNN=convolutional neural network; MLP=multilayer perceptron; AUC=area under the curve which defines the probabilty that a model ranks a random positive sample more higly than a random negative sample and its value range from 0 to1. (*Adapted from Mei et al., Nat Med, 2020)*

Very recently, an AI algorithm (resulting in a neural network model) has been developed to support these methods, integrating CT images, clinical history, testing, symptoms and laboratory findings[33]. It has been demonstrated that this neural network presents more sensitivity and specificity (84.3% and 82.8%) than models based either only on CT-scans (83.6% and 75.9%) or clinical data (80.6% and 68.3%) (figure 9)[33]. Based on these evidences, this algorithm may be used for the rapid detection of CoV-2 infected individuals, before the results of molecular testing.

Conclusions

As Covid-19 pandemic has been not eradicated yet and is rapidly climbing worldwide, fast Covid-19 diagnosis is mandatory, to isolate patients and related contacts. A plethora of methods are currently being implemented, demonstrating how different research and diagnostic expertise may converge on a common field. From standard RT-PCR methods to the last frontiers of CRISPR/Cas technology and AI, a tremendous effort is currently ongoing to allow the fast and confident identification of CoV-2 infected patients, a prerequisite for the setting of proper therapeutic protocols.

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