An Analysis of the Current Diagnostic Technology for Quick Test for SARS-CoV-2

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Abstract

SARS-CoV-2 is a beta coronavirus that first emerged in January 2020, in China. Due to the incubation nature of the virus, the early diagnosis of infection has become increasingly important for the disease containment process. This review essay will examine the currently employed diagnostic technologies such as CRISPR, qRT-PCR, ddRT-PCR and isothermal amplification in close detail. The main focus of this review essay is on the latest diagnostic technologies, their operative principles, real-life application on COVID-19 and a comparison on the tests' effectiveness and availability.

Introduction

The COVID-19 pandemic currently has more than 15 million confirmed cases; with almost 600 thousand deaths as of July 2020. The disease was first discovered in January 2020; where it is known to have originated from Wuhan, China. The disease spread rapidly to all parts of the world; effectively affecting and disrupting the day-to-day lives of billions of people [1]. In March 2020, the World Health Organization (WHO) declared the COVID-19 disease as a global pandemic.

The COVID-19 disease is caused by a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); a single-stranded RNA virus that belongs to the Coronaviridae family. The virus commonly affects mammals and avian species. There are seven types of zoonotic coronaviruses that have the ability to transmit from animals to humans; four of the zoonotic virus species (229E, NL63, OC43, HKU1) cause mild common cold symptoms for humans, but the other three beta species (SARS-CoV, MERS-CoV and SAR-CoV-2) can cause a respiratory infection, which leads to viral pneumonia and acute respiratory distress syndrome (ARDS) [1]. In addition to respiratory symptoms, uncontrolled SARS-CoV-2 infections can trigger a cytokine storm; whereby cytokines and chemokines (tumour necrosis factor-α, IL-1β and IL-6) are overproduced by the immune system, this results in inflammation and multiorgan damage [2]. There are numerous trails running worldwide that aim to produce preventive vaccines for COVID-19. Hopefully, a successful candidate can be brought to market in the next few months.

The SARS-CoV-2 transmits person to person via respiratory droplets and fomite transmission. When aerosolized, it can spread long distances [3]. Notably, the asymptomatic transmission has been recorded; meaning that the virus can spread during the incubation period, from the time of exposure and to symptom onset. The mean incubation period for COVID-19 is 5.2 days, yet extreme cases of 19 days are rare but also described [4].

Once the virus enters the body, the Spike protein (S protein) will bind with the angiotensin-converting enzyme 2 (ACE2) on the surface of type II pneumocytes, lymphocytes and epithelial cells of the mucosa and the renal distal tubules [5]. While the S1 subunit facilitates binding with ACE2, the S2 subunit mediates with the fusion of viral membrane and host cell membranes. Once the viral genome RNA is released into the cytoplasm, it gets translated using the host cell's ribosomes and goes through proteolysis to create non-structure proteins such as RNA-dependent RNA polymerase (RdRP) that replicates viral RNA [1]. The RNA codes for other structural proteins (S, E, M, N) and accessory proteins after translation become new copies of the SARS-CoV-2 that are assembled in the host cells, ready to infect more cells [6].

The early symptoms of COVID-19 resemble that of normal flu. Therefore, it is difficult to distinguish it from normal flu without the help of specific diagnostic methods. The four current testing methods of COVID-19 include:

1. Blood tests that focus on the levels of alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and troponin. All of them are indicators for organ damage.

2. Chest CT scans that focus on areas of ground-glass opacity which shows lung damage.

3. SARS-CoV-2 nucleic acid tests that examine the infection on a cellular level.

4. SARS-CoV-2 protein detection using Enzyme-Linked Immunosorbent Assay (ELISA).

There are certain limitations within the blood test and chest scans. The tests are only effective during the late stages of a COVID-19 infection where certain markers appear due to damaged vital organs. Patients at this stage are harder to cure and might have already spread the virus during the asymptomatic transmission phase. As such, early detection methods that focus on nucleic acids and proteins are essential for stopping the spread of the virus in its early stage. Protein or antibody tests are used to detect immunoglobulin G and M (IgG and IgM) from the human serum of COVID-19 patients using ELISA [7]. As most antibody tests are in development, this review essay will be mainly based on the mature nucleic acid tests which include CRISPR, qRT-PCR, ddRT-PCR and isothermal amplification.

Genomic sequence and proteins of SARS-CoV-2

The principle of nucleic acid testing is based on the detection of SARS-CoV-2 specific genome sequences. Therefore, an overview of the genome and proteins it encodes is essential for understanding the nucleic acid testing.

SARS-CoV-2 has enveloped, nonsegmented positive and single-stranded RNA, it is 29,811 nucleotides long, broken down as follows: 8,903 (29.86%) adenosines, 5,482 (18.39%) cytosines, 5,852 (19.63%) guanines, and 9,574 (32.12%) thymines [8].

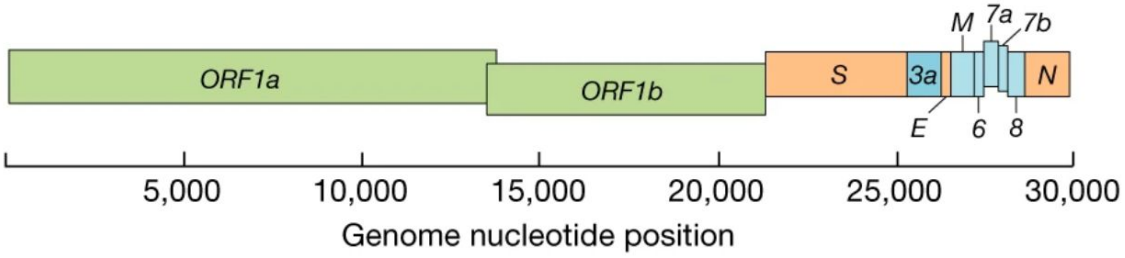

Figure 1: Genomic sequence of SARS-COV-2

There are six major open reading frames(ORF) of the SARS-CoV-2 genome (which are ORF1a, ORF1b, S, E, M and N), and there are five more accessory ORFs as can be seen in fig 1 (ORF3a, ORF6, ORF7a, ORF7b and ORF8).

The first ORFs from the direction of 5' to 3' are ORF1ab and the two together constitute more than two-thirds of the whole genome (marked as green in figure 1). The two ORFs encode 16 non-structure proteins(nsps) that all have different objectives such as preventing the host cell from producing antiviral proteins(nsp1), camouflaging viral RNA(nsp16) or controlling the movement of molecules in and out the host cell nucleus(nsp9). Noteworthily, the RNA-dependent RNA polymerase(nsp11), serves the essential purpose of making RNA copies.

The S, E, M, N genes are responsible for coding specific structural proteins (marked as orange in figure 1), which are spike protein(S protein), an envelope protein(E protein), membrane glycoprotein(M protein) and nucleocapsid protein(N protein) respectively. S protein, as mentioned in the introduction mediates the binding with ACE2 receptor on the host cell and eventually the entry of the virus; E protein is a small, integral membrane protein involved in the assembly, budding, envelope formation, and pathogenesis of the virus life

cycle[9]; M glycoprotein is in charge of virion assembly; N protein binds with the viral RNA and leads to the formation of the helical nucleocapsid.

Last but not least, the accessory genes are responsible for encoding accessory proteins ORF3a, ORF6, ORF7a, ORF7b and ORF8 (marked blue in figure 1). Although accessory proteins have been viewed as dispensable for viral replication in vitro, some have been shown to play an important role in virus-host interactions in vivo[10].

qRT-PCR

qRT-PCR (Real-Time Quantitative Reverse Transcription PCR) is a major development in the PCR technology that can be used towards the detection and measurement of RNA products[11]. It is used in a variety of applications including gene expression analysis, pathogen detection and disease research[12]. In this section, the application towards pathogen detection, specifically SARS-CoV-2 detection, will be discussed in detail.

In contrast to the conventional PCR that only allows researchers to interpret the presence of RNA sequence at the end, qRT-PCR adapts a fluorescent signal which enables observations to be made as the reaction progresses. The fluorophore, which reports fluorescent signals, is attached to the 5' end of a target-specific probe. At the same time, a quencher attaching to the 3' end of the probe is used for the silencing of the signal when at proximal to the fluorophore; Once the quencher is not near a fluorophore, the signal can be observed.

The first approach to qRT-PCR is designing and preparing the probe and primers. Corman et al. aligned and analyzed the viral genome sequences to design a set of primers and probes for SARS-CoV-2. They discovered three regions that had conserved sequences: (1) the RdRP gene (RNA-dependent RNA polymerase gene) in the open reading frame ORF 1 ab region, (2) the E gene (envelope protein gene), and (3) the N gene (nucleocapsid protein gene)[13]. Thus, these three regions are marked as target genes that are amplified in most nucleic detection processes.

qRT-PCR is done in a series of steps. The first step is putting the isolated RNA sample, taken from a swab test of a patient, in a master mix that consists of reverse transcriptase, forward primer, reverse primer, Taqman probe, free nucleotides, and DNA polymerase. During the reverse transcription process, a primer binds with the target gene, then the reverse transcriptase makes cDNA. Next, the initial denaturation is applied to the RNA-cDNA hybrid, where the temperature is raised to 95°C causing the two strands to separate[11]. After that, the reaction temperature is lowered to 58°C, allowing primers and Taqman probes to anneal to the targeted single-stranded DNA. In the next step, the temperature stays stable while DNA polymerase adds free nucleotides to the 3' end[11]. During

this process, the bond between the fluorophore and the 5’ end of the TaqMan assay is cleaved by DNA polymerase, thus the fluorophore emits fluorescent signals that can be detected with special instruments. This briefly summarises one cycle of the qRT-PCR; multiple cycles are done to achieve the desired load of target DNA.

The graph of a positive qRT-PCR test is an exponential growth figure, as the number of amplicons(fluorophore) doubles with each cycle of PCR (shown in fig. 2). The number of cycles required to pass a given threshold depends on the amount of starting template. In this way, real-time PCR can be quantitative.

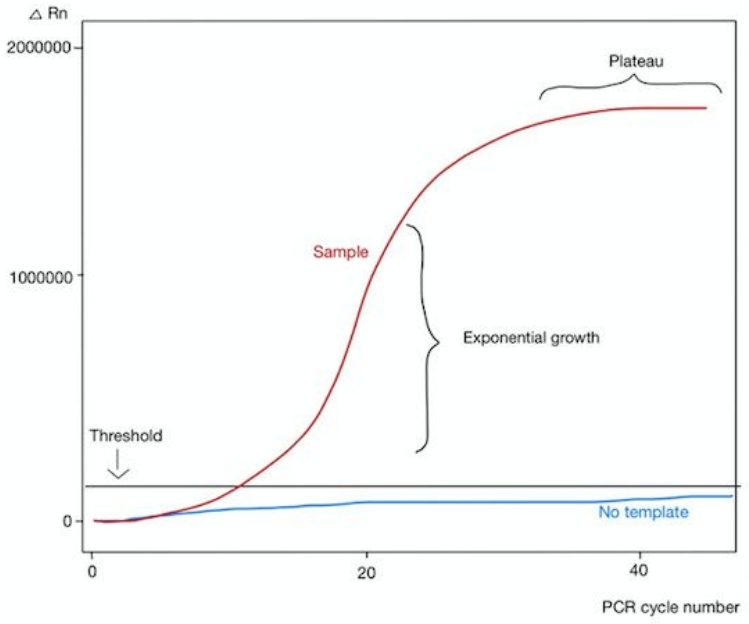

Figure 2: Real-time PCR exponential amplification demonstration

qRT-PCR is easy and fast to develop and has considerable accuracy for testing. This makes it the most widely used technology for testing. However, there are issues that have arisen with qRT-PCR. First, the results of PCR can take days to come out. Second, qRT-PCR can not identify past infections since it cannot detect proteins or past immune response.

ddRT-PCR

Digital droplet reverses transcription PCR (ddRT-PCR) is a digital PCR method using a water-oil emulsion droplet system. RNA samples are put into the water-oil emulsion to be fractionated into 20,000 nanoliter-sized droplets, and reverse transcription and amplification are carried out in every droplet[14]. The process of amplification is very similar to that of qRT-PCR, every droplet is placed into a master mix that contains various enzymes, free

nucleotides, primers and Taqman probes[14]. The fluorescent signal is also utilized in ddRT-PCR to determine if a target sequence exists in the samples[14].

Sample partition is essential to ddRT-PCR. In traditional RT-PCR, a single sample generates one result, but in ddRT-PCR one sample offers thousands of independent measurements. Thus it gives thousands of data points, bringing the power of statistical analysis inherent in digital PCR into practical application. Furthermore, this technique has a smaller sample requirement than other commercially available digital PCR systems, reducing cost and preserving precious samples[14]. ddRT-PCR has been proved to be useful not only for viral diagnostics but also for quantification of the virus in research into treatment development and vaccine production, which would become very useful in later stages of the pandemic. Nevertheless, there are disadvantages to the use of ddRT-PCR. Firstly, it required specific instrumentations, making it hard for untrained individuals to operate. Secondly, results take hours to days to come out, making it unable to accommodate high sample throughput and slowing the process of quarantine and hospitalization. Thirdly, like qRT-PCR, nucleic acid tests are unable to detect past infection.

isothermal amplification

Isothermal amplification is another detection method for SARS-CoV-2. This technology can be conducted at a single temperature environment while providing a similar accuracy to the PCR testing. Isothermal amplification includes recombinase polymerase amplification, helicase-dependent amplification, and LAMP (loop-mediated isothermal amplification)[15]. Successful RT-Lamp applications have been performed by Zhang *et al*. and El-Tholoth *et al*., where the technology has not only successfully detected samples with SARS-CoV-2, but also with the exhibition of 10-fold higher sensitivity compared to PCR [16-17].

LAMP utilizes four to six primers to bind to distinct regions on the target genome. In a four primer scenario, there are two inner primers, including FIP (forward inner primer), BIP (backward inner primer), and two outer primers[15]. As shown in figure 3, FIP hybridizes to F2c in the target DNA and initiates complementary strand synthesis. Outer primer F3, slowly hybridizes to F3c in the target DNA and initiates strand displacement DNA synthesis, releasing a FIP-linked complementary strand, which can form a looped out structure at one end. The same procedure happens with BIP and outer primer B3[15]. This leads to the production of a dumbbell-shaped sequence DNA template, which serves as the starting material of LAMP cycling. In the cycling step, FIP and BIP sequences hybridize to the F2c and B2c sequence of the dumbbell structure and exponentially amplify the sequence by recombinase polymerase amplification(Fig.3). The reaction occurs in <1 h at 60–65 °C with an analytical limit of detection of ~75 copies per μL[15].

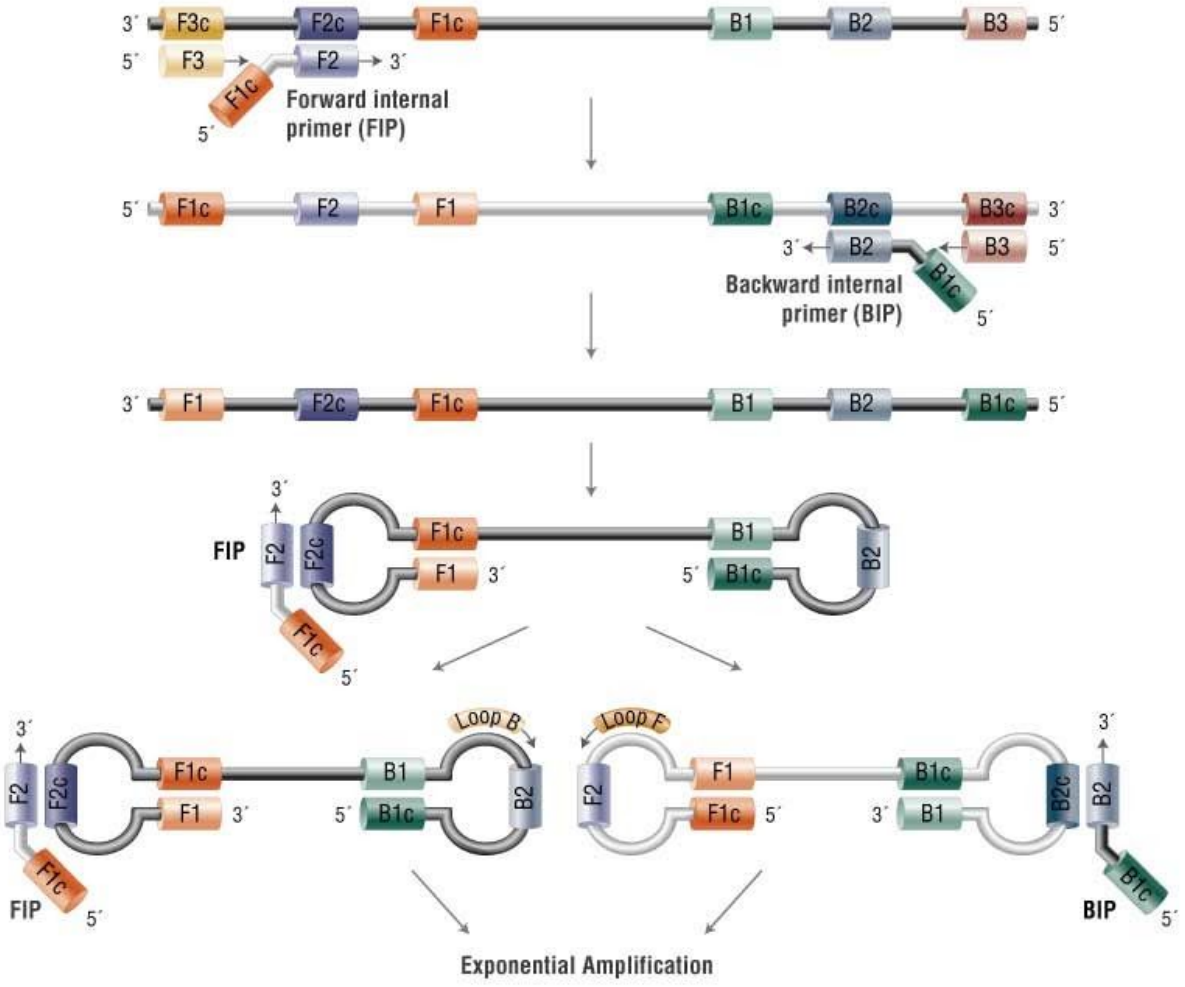

Figure 3: Schematic representation of the mechanism of LAMP. Figure is taken from the journal "Loop-Mediated Isothermal Amplification",New England Biolabs Inc.

The approach is simple to operate, gives faster results, easy to visualize for detection and does not need a thermocycler. Also as said above, multiple LAMP developments are proved to give a more sensitive result than conventional PCR. However, the drawbacks to LAMP are the challenges of optimizing primers and reaction conditions, therefore, developing a LAMP assay takes more time and resources compared to PCR.

CRISPR

Clusters of regularly interspaced short palindromic repeats (CRISPR) are a technology derived from bacteria's defensive mechanism against bacteriophages. Special Cas-proteins are developed to recognize and cut foreign DNA or RNA resulting in the malfunction of bacteriophages' replication machinery. Cas9 was first discovered and has

since been applied for gene editing purposes. Later, Cas13 and Cas12 have been discovered for their ability to facilitate the diagnosis of single-strand RNA(ssRNA) virus and DNA virus infections respectively. As SARS-CoV-2 is a ssRNA betacoronavirus, the SHERLOCK technology that involves the use of Cas13 enzyme will be observed in detail.

SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) is applied for the purpose of SARS-CoV-2 detection. The procedure presents three steps; firstly, the nasopharyngeal or oropharyngeal swab from individuals is extracted and purified. These samples are put into a tube with buffers, free nucleotides, primer pairs and RNA polymerase for performing isothermal amplification[18]. In this first step, as shown in figure 4, the viral ssRNA is amplified with recombinase polymerase amplification (RPA), which is a common procedure for LAMP. This step is followed by the detection of viral RNA using Cas13 (shown in the Cas13a system in fig. 4). The amplified sample is mixed together with special Cas13 proteins that are engineered to have a guiding RNA sequence matching the S and Orf1ab gene of SARS-CoV-2[18]. Once a Cas13 molecule binds with the target sequence, the protein will start collateral cleavage of non-specific RNA, including the single-stranded DNA probes added into the mixture (shown as step 2 of fig. 4). The probes (shown as cleavage reporter in fig. 4) change conformation and can emit fluorescent signals. Finally, in the third step, the result is visualized using a computer or naked eyes to detect fluorescent signals. Another method for detection is lateral flow visual readout. For the lateral flow readout, uncleaved reporter molecules are captured at the first detection band (control band), whereas the Cas13 cleaved reported molecules change conformation and bind at the second detection line (detection band)[18]. Therefore, by determining which line the reporter probes are on, the presence of SARS-CoV-2 can be acknowledged.

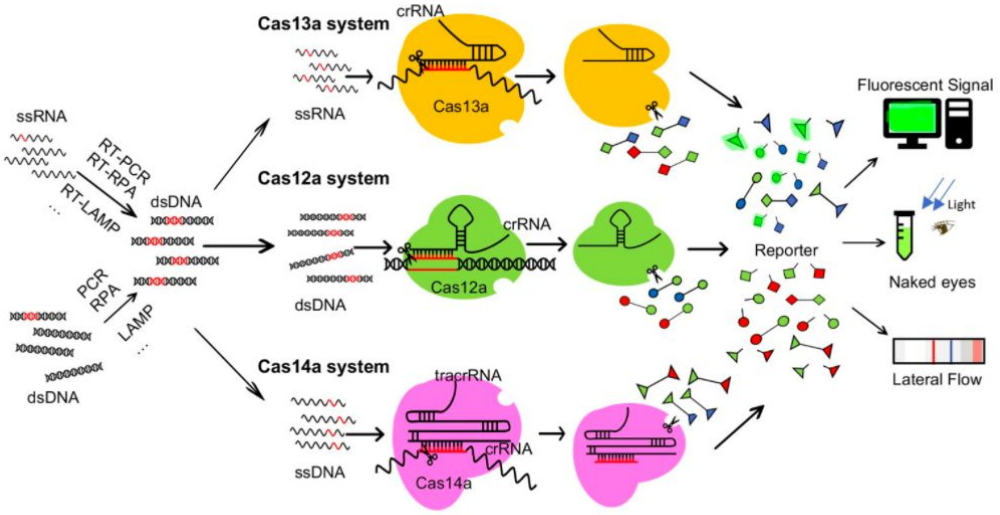

Figure 4: Schematic procedure of CRISPR. Figure is taken from the journal "Next-generation pathogen diagnosis with CRISPR/Cas-based detection methods". By Xinjie Wang ,Xiaoyun Shang and Xingxu Huang. July 20, 2020.

Figure 5 is a real-life demonstration of the lateral flow readout. As seen in the figure, detection for S gene can be made when concentration is 20aM, however, the Orf1ab gene concentration has to exceed 200aM to allow an accurate observation. Nevertheless, SHERLOCK has demonstrated exceptional sensitivity, specificity and speed compared to PCR testing and isothermal amplification diagnosis. However, some drawbacks for this technology are its incapability of detecting previous infections and its long and complicated development stage.

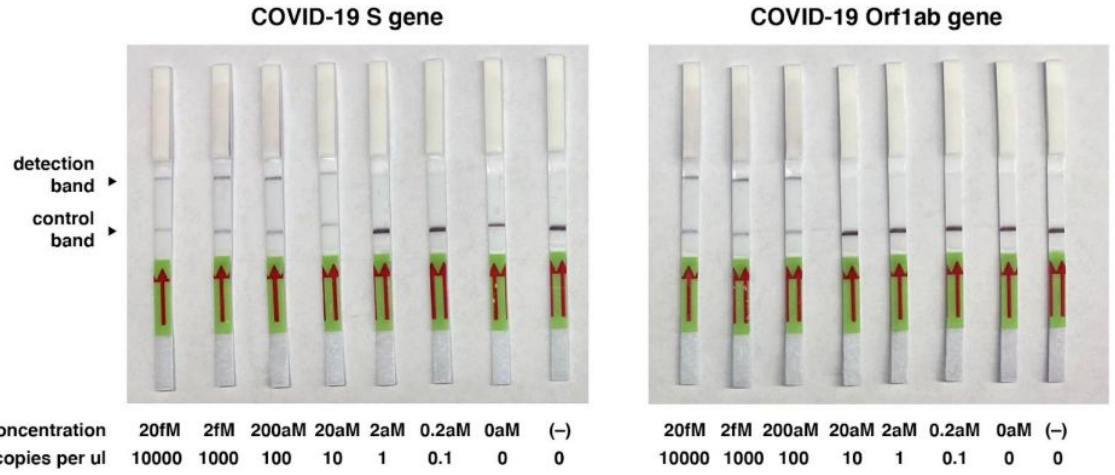

Figure 5: Demonstration of the sensitivity of SHERLOCK testing on lateral flow readout.Figure is taken from the online file "A protocol for detection of COVID-19 using CRISPR diagnostics". By Feng Zhang, Omar O. Abudayyeh and Jonathan S. Gootenberg.

Conclusion

COVID-19 has a period of incubation where infected patients show no visible symptoms but are able to transmit the virus to another healthy individual. Given the nature of the disease, detecting viral infection at an early stage proves to be critical in controlling the pandemic. This review essay examines three different types of diagnostic technologies: PCR, isothermal amplification and CRISPR. The three technologies all take the genetic approach of detecting SARS-CoV-2, which means their working principles depend on finding viral RNA in human cells.

A complete summary of the compare and contrast of the three technologies is shown in table 1 below.

Table 1: A summary of the pros and cons of qRT-PCR, ddRT-PCR, isothermal amplification and CRISPR

	qRT-PCR	ddRT-PCR	Isothermal amplification	CRISPR
Pros	- It is easy and fast to develop. Primer and probes only take a few weeks or months to design, thus the technology can be quickly put into application. - The test is relatively accurate. In a study published in *Radiology,* which was titled "Correlation of Chest CT and RT-PCR Testing in Coronavirus Disease 2019 in China," the accuracy of qRT-PCR is around 97%[19]. - Allow some point of care (POC) options.	- ddRT-PCR allows absolute quantification, which allows the exact number of viral RNA copies to be determined. - ddRT-PCR has a higher accuracy when compared to qRT-PCR (around 98% accuracy). - It has a maximum of 500 times sensitivity to low analyte environments than qPCR[20]. - Allow some point of care (POC) options.	- Isothermal amplification is highly specific. It utilizes multiple primers targeting different regions on the genome. - More sensitive than conventional PCR tests. It is found to be 10-100 fold more sensitive than PCR[21]. - Amplification occurs at a stable temperature, therefore, thermal cyclers are not needed. This cuts the budget.	- CRISPR, specifically, SHERLOCK produces high specific, accurate, and rapid results. Some methods can even detect as low as 2 samples of SARS-CoV-2 copies[22]. - POC options are available and are in development right now.
Cons	- PCR is a timely process and results usually come out in hours and even days. - PCR requires specific instrumentation that is hard to perform outside a laboratory or professional setting. - PCRs are unable to detect past infections		- isothermal amplification is unable to detect past infections - It is hard to develop as multiple primers need to be designed.	- The methods are undergoing development, and it is not yet validated yet.

POC (point of care) testings are simple kits that can be used outside of laboratory settings. These chemical kits are usually easy-to-operate and give rapid results. POC testing can be a great next-step for the diagnosis of SARS-CoV-2. However, up to this moment, POC testing is only available in research settings but not for commercial reasons.

Similarly, CRISPR testing, specifically SHERLOCK testing, is not available on the market as it is still in development. Nevertheless, this gene-editing procedure shows promising accuracy and speed potential that ensures efficient future use for viral diagnosis.

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