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SHERLOCK and DETECTR CRISPR-CAS systems as better diagnostic tools for COVID-19

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ABSTRACT: SARS-CoV-2, the mighty manslayer, responsible for COVID-19, has currently killed over 1.54 million people worldwide and 141,000 in India alone. It has affected around 67 million people globally and 9.68 million in India. It has quarantined the whole world. Doctors and scientists are working around the clock to save the world from this deadly virus. Since the number of patients is increasing rapidly, it is essential to test as many suspects as possible. But with the diagnostic tests that are being used currently, the polymerase chain reaction, antibody detection (Serological tests), Rapid Diagnostic tests (RDT), antigen tests and Isothermal Amplification assays are time consuming and there is a high chance that the test might come back with the wrong results. SHERLOCK and DETECTR are CRISPR-based diagnostic tool that were recently worked upon and showed very promising results. The test results come back in less than 40 minutes and the tests are far more accurate than all of the current diagnostics which makes them far more efficient than the others.

Keywords: COVID-19; CRISPR; DETECTR; SARS-CoV-2; SHERLOCK.

1. INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has been affecting the world since December 2019. It was first identified in Wuhan, China and since then, it has been on a continuous force of invasion to humanity. It is known to cause the "Coronavirus Disease 2019" or COVID-19. SARS-CoV-2 has been known to cause many complications including pneumonia, viral sepsis, acute respiratory distress syndrome, kidney failure, cytokine release syndrome etc. that are accountable for various symptoms or pathological changes like spike in fever, dry cough and fatigue being the most common ones. Since the outbreak, the whole world has been working to find a cure for COVID-19. Many of the research institutes have also been working on various possible diagnostic tools so as to speed up the detection of SARS-CoV-2 [1, 2]. One such attempt to redefine the process of diagnosis and eliminate the time constraint was to use CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) which is a universally acclaimed genome-editing tool when paired up with the "CRISPR Associated Protein 9" (Cas9). "Distant cousins" of Cas9, Cas12a and Cas13 have now been worked upon and found to be two of the most efficient tools of diagnosis. DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter), a CRISPR Cas12-based diagnostic tool (discovered by researchers of Mammoth Biosciences) and SHERLOCK (Specific High-

sensitivity Enzyme Reporter unlocking), a CRISPR Cas13-based diagnostic tool (discovered by McGovern Institute) are the two recently found diagnostic tools based on CRISPR which is time efficient and scientifically more accurate. SHERLOCK refers to the method using a CRISPR enzyme for collateral detection with any pre-amplification of RNA whereas DETECTR refers to the specific instance of using Cas12a collateral detection after pre-amplification by RPA (Recombinase Polymerase Amplification). The article firmly projects on how these two CRISPR-based tools function and achieve more credibility than the currently used diagnostics kits [3].

2. COVID-19 AND ITS PATHOPHYSIOLOGY

Coronavirus disease-19 (COVID-19) is a potentially fatal disease caused by the SARS-CoV-2. An initial outbreak turned epidemic further turned pandemic ultimately lead to mass isolation or rather a quarantine, due to the person-to-person transmission of the infection. Some of the most common symptoms include fever, cough and lethargy on the onset of the COVID-19 illness. With a median of 14 days, there are about 6-41 days period between the onset of the virus and death of the infected. The symptoms further elevate to lymphopenia, haemoptysis, fibrosis, dyspnoea, diarrhoea added on to which is the increase in sputum production [1, 2]. One huge difference between SARS and MERS vs. SARS-COV- 2 is that the latter developed gastro-intestinal symptoms such as diarrhoea while the former had highly rare cases with gastro-intestinal symptoms. The virus has spikes that are made up of proteins called the S-proteins. This protein is a key that attaches the virus to a human [4, 5]. The human alveoli have 3 types of cells: Type 1 cells that are squamous epithelial cells for gas exchange, Type 2 cells that are surfactants to absorb the water molecules so that the alveoli doesn't collapse and Type 3 Macrophages to kill pathogens if entered. The Type 2 cells i.e., the surfactants have a protein receptor called ACE2 (angiotensin converting enzyme-2; helps to maintain blood pressure). This ACE2 acts as a receptor where the S-protein binds and thus the virus enters the Type-2 cells.Once the virus enters the cells, it replicates and makes multiple copies of itself. Initially only the viral RNA enters the cell and hijacks its machinery. The ribosomes present in the cell, translate the viral RNA and produce 2 proteins. Further it translates the RNA this time in 3'-5' direction and synthesizes all the components of the virus such as the envelope, spike and assembles all the components into viruses and cell lysis takes place [5, 6]. Since the ACE 2 was responsible for the blood pressure maintenance, after the lysis there is a sudden drop in blood pressure and the blood vessels are dilated [7]. With millions of viruses in the alveoli, it causes irritation which further develops into a cough wherein a bunch of these viruses are coughed out which can be transmitted to other people. The alveoli that contains all the viruses prepares itself to burst and when it does so, it releases pro-inflammatory chemicals causing redness, swelling, pain, etc. apart from this, these chemicals make the blood vessels porous and permeable enabling the plasma and WBC's to flow between the vessel and alveoli and also into the alveoli trashing the gaseous exchange system leading to what is called "Acute Respiratory Distress Syndrome". This is localized inflammation. When these chemicals enter the bloodstream and affect the whole body, it elevates to what is called a systemic inflammation resulting in septic syndromes followed by organ failures ultimately resulting in death [6, 7]. Figure 1 depicts the process of infection by SARS-CoV-2.

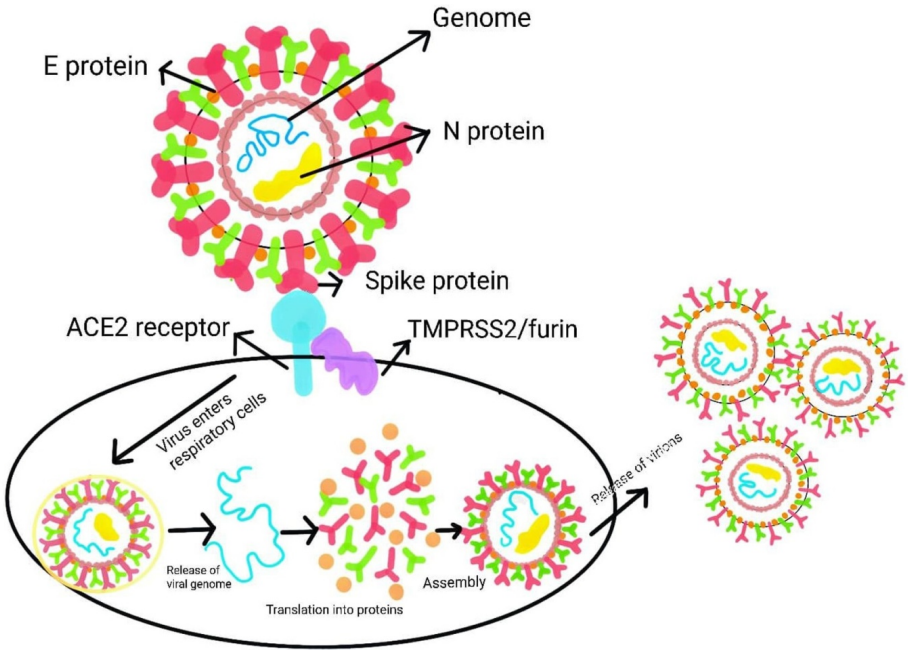

Figure 1. SARS-CoV-2 and its pathophysiology.

3. GENOME ANALYSIS OF SARS-COV-2

All corona viruses have a genome size ranging from 26,000 to 32,000 including a variable number of open reading frames or ORF's (usually 6 to 11 in number). The first ORF represents 67% of the viral genome that encodes for non- structural proteins whereas the remaining ORF's code for accessory proteins and majorly structural proteins including the spike surface glycoprotein (encoded by the S-gene), small envelope protein (encoded by E-gene), matrix protein (encoded by M-gene) and nucleocapsid protein (encoded by N-gene). On further analysis and comparison with SARS-CoV and MERS-CoV, it was identified that they are almost identical with only five nucleotide difference in the genome of approximately 2.9 kb nucleotides [2, 8]. SARS-CoV-2 was inferred to have 14 ORFs encoding 27 proteins. Also, an examination of amino acid substitution in SARS-CoV-2 (when compared to SARS-CoV) showed that there was a substitution of a total of 380 amino acids. Most of these amino acid substitutions were found in the structural proteins. This indirectly implies that the little mutations that distinguish SARS-CoV-2 from SARS-CoV are in the genes E, N, M and S. There were no substitutions of amino acids in the non- structural proteins [9-11].

4. CURRENT MEDICAL APPROACH

The treatment is based on oral drug intakes. Anti-viral hydroxychloroquine with regular doses of azithromycin are prescribed as the first line medication. Hydroxychloroquine is known to change the pH of endosomes thereby preventing the entry of the virus. It inhibits the infection of cells by SARS-CoV-2. Azithromycin belongs to class of macrolide antibiotics which prevents the currently suffering patient from getting any other bacterial infection. Other complimentary doses include vitamins C, B-complex and zinc supplements which are carried out till the patient is out of threat. These supplements act as immunity boosters. Doses of Oseltamivir and Remedesivir are then followed as the second line of medication. These drugs are known to inhibit viral RNA synthesis thereby inhibiting viral replication [12-14].

5. PREVAILING METHODS OF DIAGNOSIS

5.1. Cobas® SARS-CoV-2 test

It is software which specifically detects SARS-CoV-2 with a full process negative control and positive control. It is a qualitative assay done with Cobas® 6800/8800 systems. The major drawback of this tool is that it is very expensive and cannot be transported very frequently due to its heavy machinery [14].

5.2. Real-time-PCR assay

It is a standard test for COVID-19 and other viruses worldwide which require huge equipments and a lot of time for the detection of the virus. Moreover its sensitivity has dropped down to as low as 66-88% thus requiring tools that are more sensitive and efficient [15, 16].

5.3. Abbott real-time SARS-CoV-2 assay

Addressing the urgent needs of the people this assay provides fully automated solution catering about the detection of around 470 patients in 24 hours [17].

5.4. Perkin Elmer SARS-CoV-2 real-time RT-PCR assay

A reliable and high quality tool used for invitro diagnosis of COVID-19. This test can be used to detect SARS-CoV-2 RF1ab and N genes in 400 μL of the sputum samples from nasopharyngeal and oropharyngneal regions.

5.5. Other methods of diagnosis include

5.5.1. Isothermal nucleic acid amplification test

Processes like -loop mediated isothermal amplification along with reverse transcription RT-LAMP combined with pH indicator allows direct detection of viral RNA by a change in colour.

5.5.2. Antibody test

Otherwise known as a serology test, tested for the presence of IgM and IgG which show up in the blood on the onset of the virus and 7–10 days after the entry of the virus respectively.

5.5.3. Radiological test

Since the studies that can be made from a CT scan such as a consolidation or a ground-glass opacity is not a unique symptom of COVID-19 alone, the result cannot be clinically accepted and also the sensitivity is a variable an in case of radiological tests [18, 19].

The various methods of diagnosis that are acceptable by the WHO are presented in Table 1.

Table 1. Various methods of diagnosis accepted by WHO for detection of SARS-CoV-2 with its manufacturer and date of acceptance.

Date listed	Product description	Manufacturer
April 3, 2020	Cobas SARS-CoV-2, a software-based detection	Roche Molecular Systems
April 7, 2020	RT-PCR assay (real time-Polymerase Chain Reaction)	Primerdesign
April 9, 2020	Abbot Real-time SARS-CoV-2 (a large scale diagnostic platform)	Abbot Molecular
April 24, 2020	Perkin Elmer Real-time RT-PCR assay (more reliable *in vitro*- assay)	SYM- BIO LiveScience

6. INTRODUCTION TO CRISPR

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a genome editing tool that was first observed in *Escherichia coli* in 1987 and was found to be functioning as an immune system in bacteria and archaea [20]. Various genomic analyses around 2000's gave a clear picture of CRISPR. CRISPR and Cas proteins together worked as an acquired- immunity system for the prokaryotic cells and protected it against viruses and various plasmids. CRISPR in prokaryotes is similar to the property of memory in human immune system. On the first invasion by a virus or plasmid, the cell keeps a part of the attacker's genome, just like a mug shot, so that it can protect itself from further attack [21]. The CRISPR-Cas protein system is analogous to the RNAi (RNA interference system). One of the major characteristics of the CRISPR system is that the repeat sequences with a constant length generally have dyad symmetry and hence form a palindromic structure. Its ability to identify specific genome sequences and edit it when in association with Cas protein makes it a very desirable tool in the field of genetic engineering and has taken the scope of genome editing to the very next level [22, 23]. Due to this very reason CRISPR-Cas9 system has been used for various research programs. For example, in December 2013, the genetic mutation of the *Crygc* gene in mice was corrected using CRISPR-Cas9 system [24]. Figure 2, illustrates the activity of CRISPR-Cas9 system which makes it a viable genome editing tool.

All this accounts for the variety of fields where CRISPR systems can be viably used. Further studies and research brought to notice the presence of Cas12a and Cas13 proteins that are very different from Cas9 but when paired up with CRISPR, it can be used in a variety of ways for detection, confirmation and analyses of the desired genes (or DNA sequences). DETECTR is the diagnostic tool based on CRISPR-Cas12a system whereas SHERLOCK is the diagnostic tool based on CRISPR-Cas13 system. The above mentioned tools are further discussed in detail [25, 26].

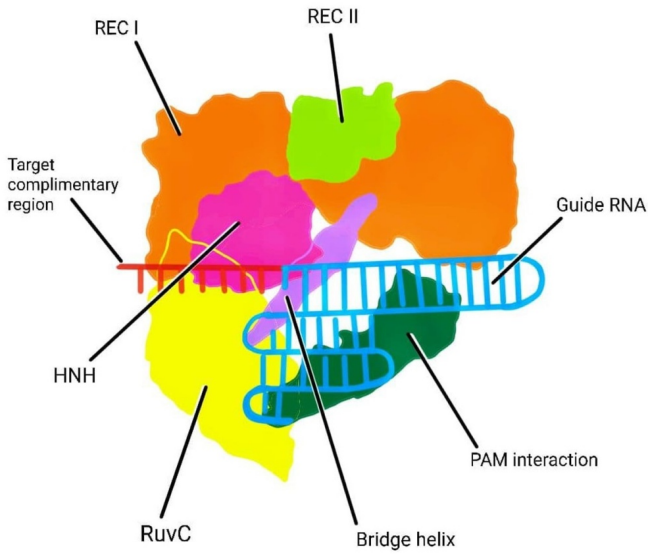

Figure 2. Cas9 complex.

6.1. General mechanism of CRISPR systems

Engineered CRISPR systems consist of two major components: the guide RNA (gRNA) and a CRISPR associated endonuclease (Cas protein). The gRNA consists of two specific regions: scaffolding that is essential for the attachment of the Cas protein and the spacer consisting of approximately 20 nucleotides that

is complimentary to the genomic target. There are two conditions that are to be fulfilled by the genomic target: (1) the target should be a set of 20 nucleotides that are specific and unique compared to the rest of the genome, (2) the target should be present immediately adjacent to the PAM (protospacer adjacent motif) sequence. The PAM sequence serves as a binding signal for Cas protein [27, 28].

The scaffolding interacts with the Cas protein to form a ribonucleoprotein. Once the PAM sequence is recognised, the Cas protein attaches itself to the PAM and the spacer is ready to bind to the target. If the spacer sequence shares sufficient homology with the target genome, the Cas protein starts functioning. In case of Cas9 it starts annealing the genome whereas in case of Cas12a and Cas13, the genome is shredded off [27-29].

The CRISPR systems of SHERLOCK and DETECTR are quite similar. The mechanism includes construction of a guide ssRNA that identifies a specific gene set that is unique to the virus in diagnosis. When the guide ssRNA binds to the set of genes, both Cas12 and Cas13 start cutting all the available nucleic acids in the system. Therefore when additional reporter RNA molecules tagged with a fluorescent dye (fluorescein amidite, FAM) are present within the system and Proteins start cutting the nucleic acids, even reporters are cut. When these RNA molecules are cut, they produce light indicating that the protein is activated and thereby concluding that the sample has the genes that are being looked for [25, 27].

6.2. CRISPR as a diagnostic tool

CRISPR is nothing but an adapted immune system of the bacteria against the viruses. The Cas proteins are CRISPR associated endonucleases otherwise known as the molecular scissors. There are 3 Cas proteins that are involved in the applications with CRISPR namely: Cas9, Cas12 and Cas13 [25]. Cas9 usually does the precise cuttings in genome editing while Cas12 and Cas13 help in the detection of genomes and also provides signals as a sign of detection making them a better diagnostic tool. This sign is produced by a process called Trans-cleavage. Cas12 and Cas13 use a guide RNA and look out for complimentary sequences of nucleic acids in the host genome. Cas12 cuts DNA while Cas13 on the other hand cuts RNA which binds to the guide RNA when it finds its complementary sequence. This cutting is known as the Cis-cleavage. While doing this the Cas proteins also switch on their Trans-cleavage which is non-specific cleavage of any nucleic acid sequence they come across. If an artificial nucleic acid sequence, often referred to as a reporter that is fluorescence quenched (FQ) with FAM (Fluorescein amidite), is added along with the Cas proteins, the non-specific cleavage of these sequences will provide a visible signal with which we can detect the presence of the viral genome as they are tagged with fluorescent dyes [23, 30, 31]. Figure 3 depicts the general workflow of CRISPR-based diagnostic tools.

Figure 3. General workflow of CRISPR-based diagnostic tools.

6.3. DETECTR- CRISPR-CAS12A based diagnostic tool

DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) is an assay that is designed to perform simultaneous reverse transcription and isothermal amplification using loop mediated amplification (RT-LAMP) for the RNA that is extracted from the nasopharyngeal or oropharyngeal swabs in a universal transport medium (UTM) [32, 33]. Since every CRISPR-Cas based system has a guide RNA, for this process, a guide RNA is designed which specifically compliments either of the four genes that code for the structural proteins like

the S-gene (spike protein), N-gene (nucleoprotein), E-gene (envelope protein) and the M-gene (matrix protein). But the designed primers targeted the N2 region N-gene and E-gene because they had the perfect PAM sequences which the other genes lacked. Once the desired gRNA is constructed, it is inserted in the sample with the Cas12a protein. The scaffolding of the gRNA binds with the Cas12a to form a ribonulceoprotein complex. Cas12a then finds PAM and attaches itself. The spacer then recognises the target sequence and attaches itself. When this is sensed by the Cas12a, it activates itself and starts working as a paper-shredder. Cas12 will start cutting all the available nucleic acids without stopping. Therefore there are RNA reporter molecules tagged with a fluorescent dye (fluorescein amidite, FAM) that produces a colour when cut. Indirect assessment is done by the cleavage of these reporter molecules and their colour emission. The sample is then added onto a flow detection system using a lateral flow strip. If SARS-CoV-2 is absent, the reporter remains intact and collects at the first detection line, the control capture line on the flow strip. If the sample is positive, the Cas-gRNA complex will cut the target and the reporter molecules. These cleaved fragments collect at a separate location, the target capture line on the flow strip. Gold nanoparticles are also used which bind to the FAM molecule on the reporter, thus generating a visual readout on the strip. This is how the test makes diagnosis simple and accurate [32, 34].

This diagnostic test is rapid (takes under 40 minutes), easy to implement and accurate. The researchers at Mammoth Biosciences have tested this tool with 36 patients affected by COVID-19 infection and 42 patients with other viral respiratory infections. This assay is faster than all the currently prescribed diagnostic tests and is also a visual alternative, making it more efficient [32, 33, 35]. Figure 4 depicts the activity of Cas12a essential for the functioning of DETECTR.

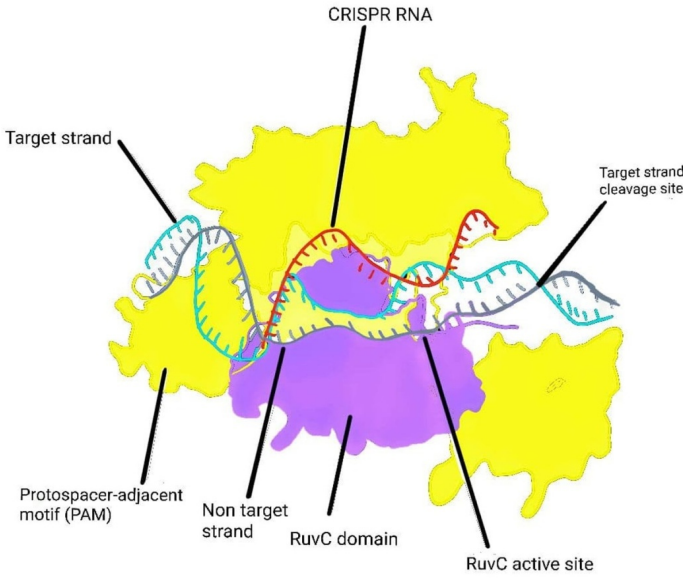

Figure 4. The activity of Cas12a.

6.4. SHERLOCK- CRISPR-CAS13 based diagnostic tool

Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK), is an in vitro nucleic acid–detection platform with attomolar sensitivity, based on nucleic acid amplification and Cas13a-mediated collateral cleavage of a reporter RNA [33, 36]. Nasopharyngeal or oropharyngeal swab sample of the person is collected. This sample is purified with all lysis reactions with proteases, lipases, etc., such that only the nucleic acid remains. The SARS-CoV-2 E-gene (envelope), N-gene (nucleoprotein) and the DNA specimen collected from the samples are amplified using any amplification technique such as RPA (recombinase

polymerase amplification) (37-42°C) or LAMP (loop mediated isothermal amplification) (62°C). To this the Master mix is added (37°C) which consists of Cas-13-crRNA, FAM-FQ reporter and T-7 transcriptase enzyme. When the Cas13 detects the presence of either the N-gene or E-gene of the SARS CoV-2, it starts to cut off every molecule that it happens to pass including the receptors. This "collateral diagnosis" provides a signal which helps to detect the presence of the viral genes through a lateral flow strip. Test kits based on CRISPR is a DNA/RNA equivalent of a pregnancy test with same principle lying behind it. The total assay reaction time is around 30-40 minutes and the net assay and result time is maximum 45 minutes [37-39]. Figure 5 depicts the activity of Cas13 essential for the functioning of SHERLOCK.

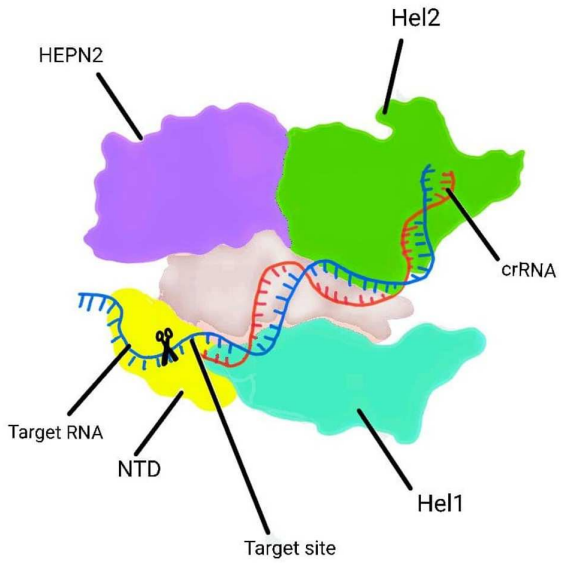

Figure 5. The activity of Cas13.

7. COMPARISON OF RT-PCR AND CRISPR-BASED MOLECULAR DIAGNOSTICS

Currently the most preferred diagnostic tool used for detection is RT-PCR. This is mostly due to the fact that the process of PCR has been used since many years for diagnosis and therefore the handling is very well known by microbiologists and pathologists. Following is the table of comparison between the most used RT-PCR and CRISPR-based diagnostic tools [3]. Figures 6 and 7 illustrate the RT- PCR method and CRISPR-diagnostic tools respectively. Table 2 shows the difference between the RT-PCR and CRISPR based detection methods.

Table 2. The difference between the presently used RT-PCR method of detection and CRISPR based detection on various basis [3].

Methods	RT-PCR	CRISPR-Based Diagnostic Tools
Specificity	Highly specific in action	Highly specific in action
Time consumed for the results	The tests results can take up to 5-6 hours to arrive.	Within 45 minutes. DETECTR takes less than 40 minutes. SHERLOCK takes up to 45 minutes.
Bulk of instrumentation	It requires many different instruments including thermal cycler and fluorimeter	No bulk instrumentation is required for either of the tools.
Cost efficiency	Due to requirement of bulky instrumentation, Cost of carrying out the procedure is very high.	Both the diagnostics are very cost efficient.
Disadvantage	Chances of false negative results are considerable, due to improper handling.	Off-targets may exist.
Target	Different labs have different targets like N-gene, Orflab etc.	SHERLOCK: N-gene and S-gene DETECTR: E-gene and N-gene

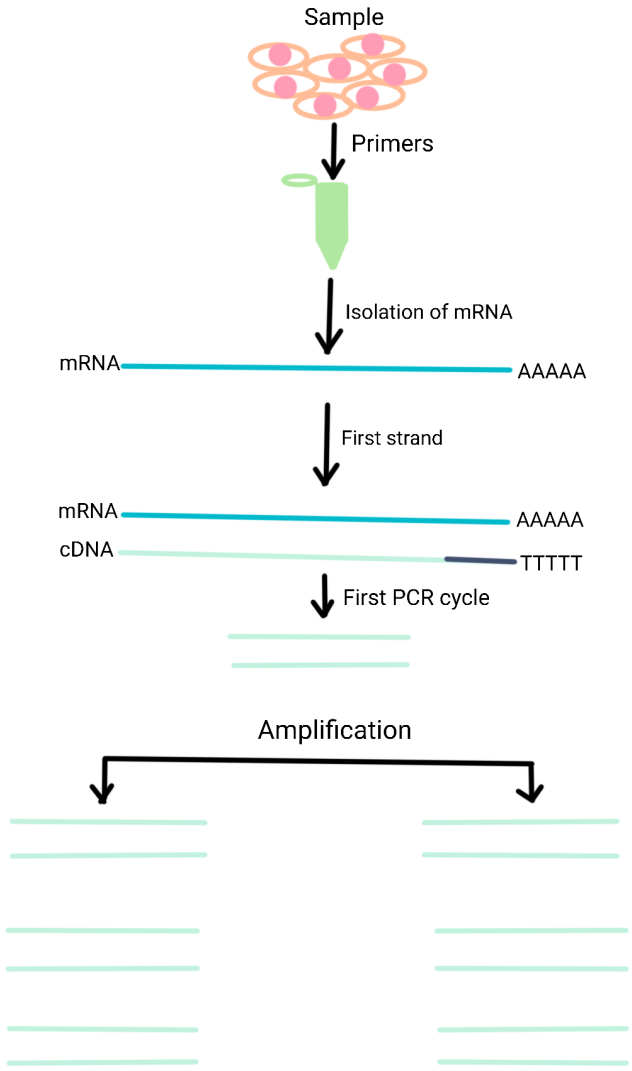

Figure 6. Real time RT-PCR assay.

7.1. LIMITATIONS

CRISPR-Cas Systems as diagnostic tools is relatively a recent concept. The F.D.A. approved the emergency use of SHERLOCK to detect the presence of SARS-CoV-2 in the U.S.A. [40]. There have also been viable clinical validations for the compatibility and accuracy of DETECTR [41]. But the biggest problem lies in the fact that these CRISPR-Cas diagnostic tools are not accessible to a lot of countries. Moreover, even if developing countries might get their hands on this technology and since it is a new process, proper training has to be arranged so that lab technicians get accustomed to the process. Based on technicality, there is a chance that off-targets may exist [3]. Another limitation of CRISPR-based diagnostic tools is that, the reaction mixtures need to be prepared which involves protein purification. Expertise in this methodology is required to properly extract and purify the desirable proteins [36, 42].

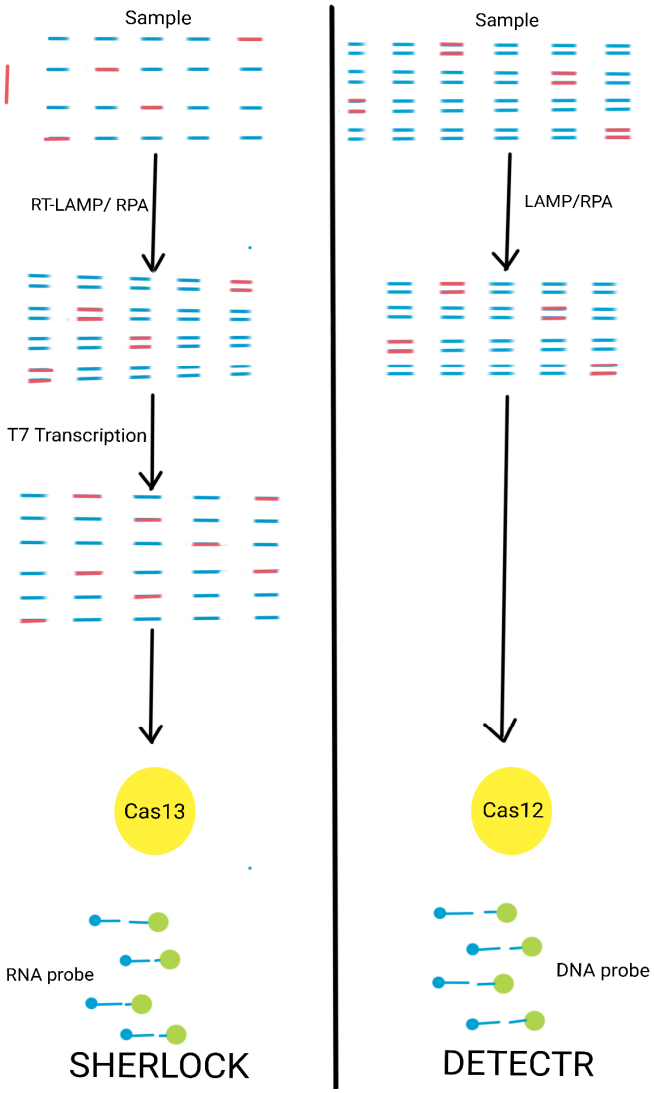

Figure 7. CRISPR-based diagnostic tools.

8. CONCLUSION

SHERLOCK and DETECTR, the two CRISPR-based diagnostics, are revolutionary in the idea of efficiency, analyses and accuracy in the field of diagnosis. These tools are very easy to handle and can be operated on a large scale basis with ease. There are no additional requirements for the whole process. The above methodology of both the processes makes it very clear that the procedures are highly specific and sensitive. Both of the two molecular diagnostic technologies, SHERLOCK and DETECTR, can be used to detect specific RNA and DNA at attomolar level (a concentration of 10^{-18} moles per litre). Two of the biggest advantages are that they can detect an early infection and are very time efficient. DETECTR gives out the test results in less than 40 minutes and SHERLOCK can give results in 45 minutes. All this makes both SHERLOCK and DETECTR very viable as a diagnostic tool, not just for the detection of SARS-CoV-2 but any viral infection in the human physiology.

Authors' Contributions: AB, EJ, and SSS planned conceptualized and designed the review article. The data collection and interpretation were done by EJ, and SSS. The article was written by EJ, and SSS with inputs from all the authors. The diagram was designed by SSS and the table was prepared by EJ. The article was amalgamated was formatted, scrutinized and finalized by EJ and SSS under the supervision of AB. All authors read and approved the final manuscript.

Conflict of Interest: The author has no conflict of interest to declare.

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