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CRISPR-based Approaches for the Point-of-Care Diagnosis of COVID19

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Severe acute respiratory syndrome coronavirus -2 (SARS-CoV-2), is a novel Betacoronavirus variant that emerged in December 2019 causing the coronavirus disease 19 (COVID19) pandemic. It is reported that asymptomatic and presymptomatic individuals can transmit the virus and this silent transmission has been a major obstacle for the control of the pandemic. To overcome this obstacle, widespread testing with a rapid turnaround time is required. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is currently the golden standard for the diagnosis of COVID19 worldwide. Even though RT-qPCR is an efficient method in terms of sensitivity and specificity, the need for elaborate instrumentation and skilled personnel restricts its widespread use. Restriction of RT-qPCR to a limited number of laboratories makes it further time-consuming. Many approaches are present to address the requirement for a rapid and accurate COVID19 diagnosis. In this review, different CRISPR-based approaches for the point-of-care diagnosis of COVID19 are compared. Among these approaches, CRISPR-FDS on-chip assay is found to be the best option as it is reported to be highly sensitive and specific, has a short turnaround time (15 min), does not need RNA isolation or special tools, and simple to perform. In terms of clinical validation, SHERLOCK, STOPCovid, and DETECTR were the most extensively studied ones and they are also reported to be highly sensitive and specific compared to RT-qPCR.

Keywords: COVID19, SARS-CoV-2, CRISPR-based diagnostics, point-of-care diagnosis, next-generation molecular diagnostics

INTRODUCTION

In December 2019, a group of patients from Wuhan, China were diagnosed with pneumonia of unknown cause [1]. Next-generation sequencing of patient specimens demonstrated that it was a novel Betacoronavirus variant [2], currently named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It is thought to have resulted from an initial zoonotic transmission event [3] and later spread through person-to-person transmission [4]. Symptoms at the onset of illness are fever, dry cough, myalgia, fatigue, and some patients have mild symptoms or asymptomatic [5,6]. After the viral transmission, individuals undergo a period of incubation. The median incubation period was estimated to be 5,1 days (95% CI, 4.5 to 5.8 days), and 97,5% of the symptomatic patients developed symptoms within 11.5 days (95% CI, 8.2 to 15.6 days) of infection [7]. Patients may show positive reverse transcription-quantitative polymerase chain reaction (RT-qPCR) test results during the incubation period, suggesting that these individuals might be infectious before becoming symptomatic [8]. Presymptomatic transmission is further supported by many case studies [9,10]. A viral dynamics study estimated the proportion of presymptomatic transmission as 44% (95% CI, 30-57%) [11]. It is reported that asymptomatic individuals have similar viral titers with symptomatic

individuals [12] and they can transmit the virus [13,14]. This silent transmission has been a major obstacle for the control of the pandemic since the emergence of the virus, proving the importance of detecting asymptomatic and presymptomatic individuals through widespread testing [15].

COVID19 has a significant mortality and morbidity burden on society and accurate COVID19 diagnosis is one of the key steps to contain the pandemic. There are currently many tests available for the diagnosis of COVID19 such as serological tests, computerized tomography (CT), and RT-qPCR tests. Serological tests, one of the first tests applied for COVID19 diagnosis, aim to detect serum antibodies against SARS-CoV-2 Spike protein. It is an important test in terms of following the immune response resulting from the infection or vaccination. However, it is not well suited for the acute phase diagnosis [16] since IgM/IgG production starts from 4 days after the onset of symptoms [17] and shows a rapid increase at 6-7th days [18]. Another diagnostic approach, CT scan is a highly sensitive alternative to RT-qPCR. It is reported in two meta-analysis that CT scan has the sensitivity of 87% (95% CI, 85-90%) [19] and 91.9% (95% CI 89.8%-93.7%) [20] compared to RT-qPCR. In a study with 1014 patients, it was reported that 60-93% of the cases had initial positive CT result before positive RT-qPCR result [21]. Despite these features, a CT scan has the disadvantage of having low specificity. According to two aforementioned meta-analysis, CT scan has the specificity of 46% (95% CI, 29-63%)[19] and 25.1% (95% CI, 21.0%-29.5%)[20].

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is currently considered the golden standard for the diagnosis of COVID19 [22,23]. However, the test has many shortcomings. First of all, it is stated that RT-qPCR has many pre-analytical and analytical vulnerabilities that can jeopardize its results [24]. Furthermore, false-negative results have been reported [21,25] which raise the suspicion of the RT-PCR test as the golden standard diagnostic tool for COVID19 [26]. Starting from sample preparation to final readout, processing of conventional RT-qPCR requires 4-6 hours [27]. However, the overall turnaround time is much longer. According to a large survey in the United States (with 19,058 responders), the average wait time for the test result was 4,1 days [28]. In Turkey, average wait time for the test result is estimated to be1-2 days (Unpublished data). This

experienced delay is thought to have resulted from high-frequency testing and limitation of RT-qPCR to central laboratories. RT-qPCR requires elaborate and expensive instrumentation and technical expertise which increase its cost and limit its widespread use. Therefore, a point-of-care diagnostic test with a rapid turnaround time remains to be an urgent requirement to contain the pandemic.

In this review, we will compare some of the many CRISPR-based approaches developed for the diagnosis of COVID19 in terms of sensitivity, specificity, and turnaround time. We will discuss the workflow of each test and try to give an idea about their applicability as point-of-care diagnostic tools.

Rapid Detection of SARS-CoV-2 Nucleic Acids

Besides the aforementioned diagnostic tests, there are many rapid COVID19 tests suitable for point-of-care diagnostics. These rapid tests include RT-LAMP tests [29], Rapid Antigen Tests [30], and CRISPR-based approaches [31]. Among these approaches, nucleic acid detection methods mainly rely on isothermal amplification methods instead of conventional PCR to free the test from the restriction of having a thermocycler. One of the rapid nucleic acid detection systems is Abbot ID Now™. It is an automated system taking advantage of isothermal amplification and approved by the United States Food and Drug Administration (U.S. FDA) [32] as a point-of-care diagnostic test. However, the overall positive percent agreement of Abbot ID Now with RT-qPCR was found to be 73.9% [33] and 75% [34] in two studies. U.S. FDA recommends confirmation of negative results with a high-sensitivity authorized molecular test [35]. Another U.S. FDA-approved rapid nucleic acid detection system, Cepheid Xpert Xpress™, had 98,9% positive percent agreement with RT-qPCR [33].

RT-qPCR

RT-qPCR is currently the golden standard for the diagnosis of COVID19 and it is the main diagnostic approach worldwide [22,23]. RT-qPCR is a technique, in which amplification and detection processes are combined in a single step with the help of fluorescent chemistry [36]. An RT-qPCR reaction is characterized by the time point when the

amplifichia of the target is first detected [37]. This time point is called as cycle threshold (Ct). When there is higher nucleic acid initially, amplification is detected at a lower Ct [38]. In RT-qPCR protocol, the viral genome is first extracted from the patient samples. Then, target viral genes are converted to cDNA by reverse transcription (RT) and amplified by polymerase chain reaction (PCR). Amplified products can be detected through various methods such as DNA binding dyes, hydrolysis probes, hybridization probes, etc. DNA binding dyes (SYBR Green I) bind to dsDNA that is formed during the reaction and they are not sequence-specific [39]. It is cheaper than sequence-specific probes but it has some specificity issues [37]. When the dye binds to primer-dimers or non-specific PCR products, it may produce false-positive results [40]. According to the United States Centers for Disease Control and Prevention (U.S. CDC) guideline, primer-probe sets targeting the Nucleocapsid (N) gene of the SARS-CoV-2 are used [41]. Other primer-probe sets recommended by the China CDC, Hong Kong University, and World Health Organization (WHO) have also been proven to have enough analytical sensitivity [42]. RT-qPCR has an analytical limit of detection (LOD) of 1,000 viral copies/mL (1 copy/μL) [42]. There are many different findings in terms of sensitivity and specificity of RT-PCR and results vary according to the chosen patient specimen. Overall, RT-qPCR testing of lower respiratory specimens (Bronchoalveolar lavage fluid and sputum) provide the highest sensitivity [43,44]. It is stated in a meta-analysis that, RT-PCR test has the sensitivity of 97,2% (95% CI, 90,3-99,7%) with sputum, 73,3% (95% CI, 68,1%-78%) with Nasopharyngeal/Oropharyngeal swab and 62% (95% CI, 54,5%-69,9%) with saliva sample [20]. False-negative results with RT-PCR are reported [21,25] and in such cases with clinical suspicion, testing with RT-qPCR for the second time or confirmation with CT scan is recommended [19,27]. Further systematic analysis and better reference standards are needed for the comparison of RT-qPCR.

CRISPR based Diagnostic Tests for COVID19

Clustered regularly interspaced palindromic repeats (CRISPR) were first identified by Japanese researchers in 1987 as "short direct repeats interspaced with short sequences in the genome of Escherichia coli" [45] and later found out to be present in many prokaryotes [46]. CRISPR and CRISPR-associated (Cas) proteins are together responsible for the prokaryotic adaptive immune system against bacteriophages and plasmids [47,48]. This bacterial defense mechanism was repurposed for many purposes including genome editing, transcriptional perturbation, and elucidation of gene function [49,50]. Among its applications, nucleic acid detection is proven to be highly sensitive and specific for the diagnosis of many viral and bacterial diseases [51-53].

There are many different CRISPR-based approaches for the diagnosis of COVID19. Each approach follows a different workflow as depicted in Figure 1. Starting from the specimen collection, different patient samples like saliva, sputum, nasopharyngeal, oropharyngeal, and nasal swabs can be chosen. Then, viral RNA must be extracted, and this extraction can be done through either conventional RNA isolation methods (like spin-column based isolation) or through simpler methods (like magnetic-bead assisted isolation). Extracted viral nucleic acid is later amplified through various isothermal amplification methods and readout is obtained with a lateral flow assay or with a fluorescent reporter. We will now discuss each CRISPR-based diagnostic approach and compare their results. Results obtained from each test can be seen in Table 1.

CRISPR-Cas12 based COVID19 Diagnostic Tests

CRISPR-Cas12 is an RNA-guided DNase that displays non-targeted strand cleavage (collateral cleavage) upon detection of the target sequence [54]. When the designed CRISPR-RNA (crRNA) matches with the targeted DNA sequence, Cas12 cleaves the target strand along with the nearby ssDNA and dsDNA oligonucleotides. [54]. This collateral cleavage activity is non-specific, and it can be detected through the cleavage of a quenched fluorophore ssDNA reporter or with a lateral flow strip.

4.1.1 STOPCovid.v1 and v2

Sherlock Testing in one pot (STOP) for COVID19 diagnosis is a CRISPR-Cas12 based approach for the diagnosis of COVID19 [55,56]. There are two versions of the test, and they differ in their RNA isolation methods. Joung et al. first developed a protocol that can be done in a single fluid handling step and named the approach as STOPCovid.v1 [56]. They demonstrated that nucleic acid amplification
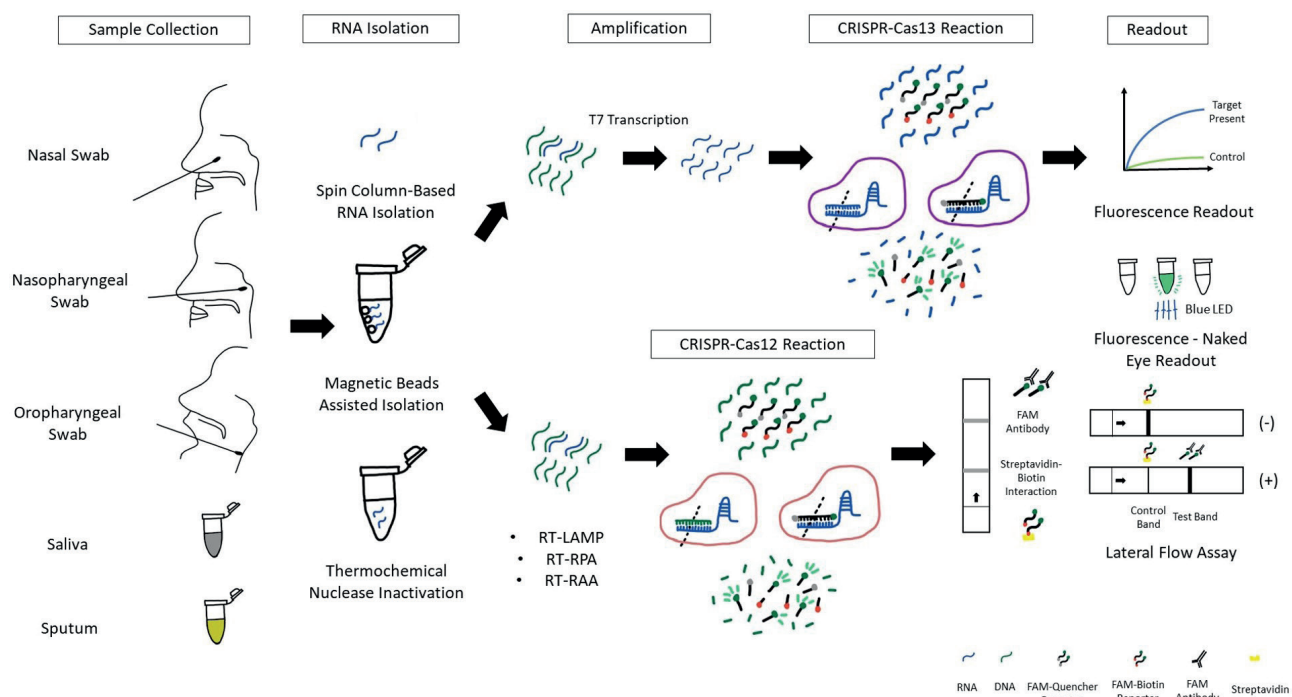
Figure 1. Different approaches are available in each step of the CRISPR-based COVID19 diagnostic tests' workflow. Please refer to the text for further information. For simplicity, Fluorophore-quencher reporter and FAM-Biotin reporter are drawn together in the CRISPR-Cas reaction. Please note that these two reporters represent different readout approaches and are not used together.

Table 1. CRISPR-based diagnostic tests. Sensitivity and specificity are results compared to the RT-qPCR test.

Diagnostic Test	Sample-to Answer Time	Limit of Detection	Tested Patient Samples	Sensitivity	Specificity	Ref.
RT-qPCR	2-8 h	1 copy/µL	-	-	-	[20,27,42]
opvCRISPR	~45 min	5 copies/reaction	50	100%	100%	[66]
DETECTR	30-40 min	10 copies/µL	83	95%	100%	[58]
RCSMS	40 min	5 copies/reaction	276	93,80%	99%	[62]
STOPCovid.v2	15-45 min	0,033 copies/µL	402	93,10%	98,50%	[55]
AIOD-CRISPR	40 min	4,6 copies/µL	-	-	-	[63]
CASdetec	1 h	10 copies/µL	-	-	-	[64]
CRISPR-FDS on Chip Assay	15 min	0,38 copies/µL	103	98,7-100%	100%	[67]
CRISPR-COVID	40 min	2,5-7,5 copies/reaction	114	100%	100%	[74]
SHERLOCK	<1h	10-100 copies/µL	534*	96%	100%	[70][72]
SHINE	50 min	10 copies/µL	50	90%	100%	[73]
Fozouni et al.	<30 min	100 copies/µL	-	-	-	[87]

RNA isolation is not included in these turnaround times except for the ones that are RNA-isolation-free (CRISPR-FDS and RCSMS). Three of the tests (CRISPR-COVID, opvCRISPR, and RCSMS) have different statements for the limit of detection as copies/reaction. *Clinical validation was performed by different research groups.

and CRISPR detection can be performed in a single step. It is a significant result because when we do not need to transfer the amplified product into a second tube for CRISPR detection, contamination risk reduces. The method consists of three steps: Lysis of the virus-containing patient sample using QuickExtract™ to release the viral RNA, detection of viral RNA using STOPCovid master mix (which includes RT-LAMP and Cas12 reagents together).

Taurine can also be added to the kit to improve the reaction kinetics. The last step is the visual readout step. A commercially available (Lateral flow dipsticks) paper dipstick can be used (which gives us a result like a pregnancy test). Both NP/OP and saliva samples can be used for the detection of SARS-CoV-2, as it was previously reported that saliva samples have similar viral loads to nasopharyngeal swabs [57].

In STOPCovid.v2, a simpler RNA extraction method with a magnetic bead is performed [55]. This method is thought to be faster and less contamination prone than the conventional RNA extraction methods. Therefore, it is a feasible option for a point-of-care diagnostic test. After the RNA extraction, the RNA genome is reverse transcribed and amplified by LAMP. Isothermal amplification is also a suitable approach for point of care diagnosis because only the presence of a heat block would be enough. LAMP operates at 55-70℃ so a thermostable Cas12 enzyme, Alicyclobacillus acidiphilus Cas12b (AapCas12b), is used in the test. The test can reliably detect 33 copies/mL, which is the one-thirtieth of the RT-qPCR method (1000 copies/mL). STOPCovid.v2 has the best limit of detection among the CRISPR-based approaches reviewed in this article. The test has been applied in 402 patient samples in multiple centers. The test has the sensitivity and specificity of 93,1% and 98,5% respectively, compared to the RT-qPCR method.

DETECTR

Broughton et al. developed a method for the detection of COVID19 through CRISPR-Cas12 collateral cleavage activity [58] and shared the protocol with the public in February 2020 [59]. The method is named SARS CoV-2 DNA Endonuclease-Targeted CRISPR Trans Reporter [DETECTR]. DETECTR received emergency use authorization (EUA) from U.S. FDA on July 9, 2020 [60].

In the DETECTR assay, the extracted viral RNA from the patient sample is amplified and reverse-transcribed into cDNA by RT-LAMP reaction at 62℃ for 20-30 minutes. If the targeted SARS-CoV-2 genes are present, Cas12 cleaves the amplified single-stranded DNA together with the nearby FAM-biotin reporter. The test result is obtained through a lateral flow strip by which we can understand if the reporter is cleaved or not. The lateral flow strip provides us a qualitative result such as positive or negative. If the targeted SARS-CoV-2 genes: E and N genes are both detected with Cas12 reaction, the assay is considered as positive. If only one of these genes is detected, the result is considered as presumptive positive. The assay took 30-40 minutes to complete, and the limit of detection was 10 copies/µL. The positive predictive agreement and negative predictive agreement of SARS-CoV-2 DETECTR relative to the CDC RT-qPCR assay were 95% and 100%, respectively, for the detection of SARS-CoV-2 in 83 total respiratory swab samples [58]. This result was further supported by a multicenter study. 378 patient samples were tested with DETECTR and positive predictive agreement of the test relative to RT-qPCR was found to be 95% [61]. DETECTR is a two-tube test, RT-LAMP and Cas12 detection are performed in different tubes. This may be considered as a disadvantage due to contamination risk.

Recently, a locally adapted variant of DETECTR has been developed and named Rapid Coronavirus-Sensitive Monitoring from Saliva (RCSMS) [62]. It is tested in 276 patients. Researchers demonstrated that a low-cost thermochemical treatment with TCEP/EDTA could be sufficient to inactivate nucleases in saliva and eliminate the need for viral RNA extraction. RCSMS was able to detect 5 copies/reaction in 40 minutes and had the sensitivity and specificity of 93.8% and 99.0% respectively, relative to RT-qPCR.

AIOD-CRISPR

All-In-One Dual CRISPR-Cas12a (AIOD-CRISPR) Assay is developed by Ding et al. and the significant difference of their approach from other CRISPR-based tests is that they combined two crRNAs to improve the sensitivity of the test [63]. Although combining crRNA1 and crRNA2 did not result in a different fluorescence signal than crRNA2 alone, dual crRNA was able to detect a lower amount of SARS-CoV-2 genomic material. It is a single tube test [like STOPCovid] that does not require a transfer process. The preferred amplification method was RPA, and the readout can be obtained through both fluorescence signal and direct observation without a device, so-called naked-eye readout. AIOD-CRISPR method has been tested for the detection of both HIV and SARS-CoV-2 viruses. When they tested detection of the SARS-CoV-2 N gene on a plasmid, visual detection was obtained in 40 minutes. There was no cross-reaction (with other coronaviruses) which shows the specificity of the method. A high concentration of ssDNA-FQ reporters strengthened the signal for detection. Researchers did not perform a clinical validation for the method.

CASDetec

CRISPR-assisted detection (CASdetec) is a method employing CRISPR-Cas12b for the diagnosis of

COVID19 [64]. The detection limit of CASdetec is 10.000 copies/mL (10 copies/μL). Researchers have reported that increasing the crRNA concentration increased the rate of reaction and resulted in an enhanced fluorescence signal. They have previously demonstrated that CRISPR was unable to detect target DNA when there was <1-10nM of amplification product [65] and preferred to amplify the genomic material with RT-RAA.

opvCRISPR

One-pot visual reverse transcription (RT)-LAMP-CRISPR (opvCRISPR) test is a sensitive method with a simplified operation that implements CRISPR/Cas12a for the detection of SARS-CoV-2 [66]. RT-LAMP is used for amplification and the readout is obtained with quenched fluorescent single-stranded DNA (ssDNA) reporter. A fluorescent signal is made visible to the naked eye with the help of blue light. The significance of the opvCRISPR method is that it is shown to be superior to colorimetric RT-LAMP techniques for the diagnosis of COVID19 in terms of sensitivity and specificity. To confirm that Cas12a cleavage improves the sensitivity of the test, RT-LAMP amplification was stopped at different time points and products were cleaved with Cas12a. It is demonstrated that within 20 minutes of amplification, amplicons were below the fluorescence threshold and could not be detected with RT-LAMP alone. But Cas12a cleavage provided enough fluorescent signal within 20 minutes, thus increasing the sensitivity of RT-LAMP, and shortening the required time. The test was able to detect SARS-CoV-2 at nearly the single-molecule level and can be accomplished in 45 minutes. The method demonstrated 100% positive predictive agreement with RT-qPCR.

CRISPR-FDS on Chip Assay

CRISPR-FDS is a method that uses Cas12a for the detection of COVID19 in patient saliva specimens [67]. It does not require a separate RNA isolation step. Instead, they optimized the lysis step to make the saliva specimen compatible with CRISPR reagents. That way, the procedure is shortened, and the test is freed from expensive reagents required for RNA isolation. Lysis of the patient sample is followed by RT-RPA amplification and a CRISPR-Cas12a reaction that can be detected with fluorescent reporters. For the interpretation of the fluorescent signal, the group devised an on-chip assay that can be read by a smartphone fluorescence reader.

To summarize the workflow, saliva samples are collected into tubes that contain lysis buffers and heated for 5 minutes. Afterward, lysed samples are added to the sample wells of the assay chip. Assay Chip contains five wells that are preloaded with premixed RT-RPA and CRISPR-Cas12a reagents. The chip is then incubated for 10 minutes at room temperature and inserted into the smartphone reader. A smartphone fluorescence reader is a 3D printed device that contains a laser diode, and the results can be visualized with a smartphone. It is a portable and inexpensive device that is suitable for point-of-care testing. Overall, the sample-to-answer time was 15 minutes which is the shortest duration compared to other nucleic acid detection methods. The limit of detection of the test was 0,38 copies/μL, which occurs to be the second-best (after STOPCovid.v2) among the nucleic acid detection methods reviewed in this article.103 clinical specimens were tested with CRISPR-FDS. Saliva samples exhibited a false negative rate of 1,3% compared to RT-qPCR. However, swab samples showed complete concordance with RT-qPCR.

The group demonstrated significant results in terms of specimen collection. They demonstrated that in early periods of the SARS-CoV-2 infection, saliva retains SARS-CoV-2 for a longer time than the nasopharyngeal sample in non-human primates.

CRISPR-Cas13 based COVID19 Diagnostic Tests

CRISPR-Cas13 is a RNA-guided RNase which displays collateral cleavage activity [52,68,69]. The main difference between Cas13 and Cas12 is that Cas13 detects and cleaves only single-stranded RNA molecules. When the designed CRISPR-RNA (crRNA) matches with the targeted single-stranded RNA sequence, Cas13 cleaves the targeted ssRNA along with the nearby ssRNA oligonucleotides. Cas13 reaction can be detected just like the Cas12 activity, with a quenched fluorophore ssRNA reporter or a lateral flow assay. As Cas12 detects ssRNA oligonucleotides, the gene of interest is first reverse transcribed into cDNA and amplified by LAMP or RPA. The amplicon is then transcribed back to RNA oligonucleotides by T7 transcriptase, so-called in vitro transcription.

SHERLOCK

Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) is a CRISPR-Cas13a based nucleic acid detection method developed by Gootenberg et. al initially for the detection of Zika and Dengue virus [68]. SHERLOCK method provides rapid nucleic acid detection with attomolar sensitivity and single-base mismatch specificity. Shortly after the emergence of COVID19, Zhang et. al published a SHERLOCK Protocol for the detection of SARS-CoV-2 [70]. The protocol consists of isothermal amplification of isolated viral RNA with reverse transcription-recombinase polymerase amplification (RT-RPA) kit, in vitro transcription of the amplified nucleic acid with T7 transcriptase, and detection of the viral RNA sequence with Cas13a. Finally, a naked-eye readout of the test result is obtained by using a paper dipstick. The test was able to detect target sequences with a concentration between 10-100 copies/µL. The test takes less than an hour to perform and does not require elaborate instrumentation. SHERLOCK became the first CRISPR-based approach to receive emergency approval from the U.S. FDA for the diagnosis of COVID19 [71] (May/2020).

In another study, the SHERLOCK protocol was clinically validated with 534 patient samples [72]. It is reported that the test was 100% specific and 96% sensitive with the fluorescence readout and 88% sensitive with lateral flow readout. The limit of detection of the set with the designed crRNAs against the N gene was found to be 42 copies/reaction, which is concordant with the SHERLOCK study. They also devised the lateral flow assay and included an internal RNase to control contamination.

SHINE

SHERLOCK and HUDSON Integration to Navigate Epidemics (SHINE) is a CRISPR-Cas13a based approach for the detection of SARS-CoV-2 [73]. It does not require prior RNA isolation. The preferred RNA extraction method is heat and chemical reduction to inactivate RNases. Clinical validation of SHINE in 50 patient samples showed 90% sensitivity and 100% specificity compared to RT-qPCR.

CRISPR-COVID

CRISPR-COVID is among the first CRISPR-based tests developed for COVID19 diagnosis [74]. It detects

the RT-RPA amplified genomic material with Cas13a Endonuclease activity. The targeted SARS-CoV-2 genomic sequence is Orf1ab, which was selected because it is conserved among reported SARS-CoV-2 genomic variants, and it does not cause a false-positive result with other microorganisms. The method is tested in 114 patient samples, of which 52 were SARS-CoV-2 positive (detected with Metagenomic Next Generation Sequencing) and 62 were negative. CRISPR-COVID was able to detect 52/52 of the positive cases and it gave negative results with 62/62 of negative cases. Using metagenomic next-generation sequencing (mNGS) and PCR as the reference, CRISPR-COVID had the sensitivity and specificity of 100%. Compared to CRISPR-COVID, the PCR test was able to detect 47/52 of the SARS-CoV-2 positive patients. Furthermore, the test took CRISPR-COVID 40 minutes (30 minutes amplification and 10 minutes of Cas13a reaction) to give a result. Whereas mNGS takes approximately 20 hours. The limit of detection of the test was reported to be near a single copy/sample. Among 10 replicates, the test was able to detect all of them with the concentration of 7,5 copies/sample and it was able to detect 6/10 samples with a concentration of 2,5 copies/reaction.

Comparison of Different Workflows

As it can be seen from Table 1, each CRISPR-based diagnostic test achieved a different outcome. Different results are present since many different techniques are available in each step of the workflow. Different methods used in each step of CRISPR-based COVID19 tests are summarized in Table 2.

Sample Collection

It has been reported that positive RT-PCR can be obtained from patient bronchoalveolar lavage fluids, nasal swabs, pharyngeal swabs, feces, and blood [75]. Viral titers of patient throat swab and sputum samples peak around 5-6 days after the onset of symptoms, reaching 104-107 copies/ mL [8]. The virus can be detected shortly after the onset of symptoms and higher viral loads were detected in the nose than in the throat samples [12]. The viral load of asymptomatic individuals was similar to that of symptomatic individuals, which suggests the asymptomatic transmission [12].

Table 2. Different workflows of the CRISPR-based diagnostic tests.

Diagnostic Test	Sample	RNA Isolation	CRISPR	Target Gene	Amplification	Detection	Ref.
opvCRISPR	NP	Not Specified	Cas12a	S	RT-LAMP	F/NE	[66]
DETECTR	NP/OP	Conventional	Cas12a	E and N	RT-LAMP	LFA	[58]
RCSMS	Saliva	TI	Cas12a	E and N	RT-LAMP	LFA	[62]
STOPCovid.v2	NP	Magnetic Bead Assisted	AapCas12b	N	RT-LAMP	F/LFA	[55]
AIOD-CRISPR	-	-	Cas12a	N	RPA	F/NE	[63]
CASdetec	-	-	Cas12b	RdRP	RT-RAA	F	[64]
CRISPR-FDS on Chip Assay	Saliva	Optimized Lysis	Cas12a	Orf1ab	RT-RPA	F-SP	[67]
CRISPR-COVID	NP/OP	Conventional	Cas13a	Orf1ab	RT-RPA	F	[74]
SHERLOCK	NP/OP	Conventional	Cas13a	Orf1ab/S	RT-RPA	LFA	[70]
SHINE	NP/OP	TI	Cas13a	Orf1a	RT-RPA	F/LFA	[73]
Fozouni et al.	-	-	Cas13a	E and N	None	F-SP	[87]

There was no clinical validation performed for AIOD-CRISPR, CASdetec, and Fozouni et al. Therefore, marked with a "-". Diagnostic tests that employ thermochemical Inactivation (SHINE, RCSMS) and optimized lysis (CRISPR-FDS on Chip Assay) for viral nucleic acid extraction do not require RNA isolation. E: Envelope Gene, F: Quenched Fluorescent Reporter, LFA: Lateral Flow Assay, N: Nucleocapsid gene, NE: Naked Eye, NP: Nasopharyngeal Swab NS: Not Specified, Orf1ab: Open Reading Frame 1ab Polyprotein, OP: Oropharyngeal Swab, RdRP: RNA dependent RNA Polymerase, RT-LAMP: Reverse Transcription loop-mediated amplification, RPA: Recombinase Polymerase Amplification, RAA: Recombinase Aided Amplification, S: Spike gene SP: Smart Phone, TI: Thermochemical Inactivation

Currently, U.S. CDC recommends nasopharyngeal specimen collection, and oropharyngeal specimens are also considered acceptable specimens [76]. This collection method may impose a risk on the healthcare worker due to direct exposure. However, please note that there is limited data regarding aerosol generation and risk of transmission during NP/OP specimen collection procedures [77].

Saliva samples offer practical and logistical advantages for the diagnostic efforts as they can be directly collected by the patient. To compare the relative diagnostic value of nasopharyngeal and saliva samples in early infection, non-human primates were infected with SARS-CoV-2 in a study [67]. Results showed that the mean SARS-CoV-2 levels (detected with CRISPR-FDS) were substantially higher and more stable for a long time in oropharyngeal saliva samples compared to nasal swabs [67]. Another study indicates that posterior oropharyngeal saliva samples had better positive percent agreement than nasopharyngeal samples [78]. There is an anecdotal example of a leukemia patient, whose nasopharyngeal swab was tested negative with RT-qPCR but positive results were obtained from saliva with the CRISPR-FDS test [67,79]. In a study, saliva samples were demonstrated to have superior sensitivity over nasopharyngeal swabs [57]. Altogether, these studies suggest that saliva can be an acceptable alternative to nasal swabs.

RNA Isolation

The majority of the nucleic acid detection methods for COVID19 require a prior RNA isolation step. Isolation of viral RNA requires commercial kits, expensive reagents, and long processing times [62]. Therefore, simpler methods for RNA isolation could be more appropriate for a point-of-care diagnostic test. There is some alternative simple to perform methods to isolate RNA. One of these methods makes use of magnetic beads [55]. Some of the CRISPR protocols do not require prior RNA isolation. These approaches consist of CRISPR-FDS, RCSMS, and SHINE. These protocols are made possible by optimizing the lysis step and thermochemical inactivation of RNases.

Amplification Methods

Isothermal amplification methods have many advantages over the conventional PCR method in terms of simplicity, rapidity, and low cost [63]. Recombinase polymerase amplification (RPA) [80] and loop-mediated isothermal amplification (LAMP), are the mainly preferred methods used in many point-of-care diagnostic tests as they don't require a thermocycler and take a shorter time. Furthermore, buffers of these amplification methods can be optimized with Cas enzymes [81] to be used in a single tube which removes the requirement of sample transfer and decrease the contamination risk. Despite their advantages, there are currently some challenges in application

such as false-positive results due to non-specific amplification [82,83].

Recombinase polymerase amplification (RPA) operates at the temperature of 37-42°C, which is an advantage over LAMP because it does not need any instrumentation for the amplification. Even holding the tube in the hand could be enough to proceed. The disadvantage of RPA is that it has support chain restrictions whereas requisite enzymes for LAMP are more readily available. Loop-mediated isothermal amplification operates at 55-70°C. Therefore, a thermostable Cas enzyme like AapCas12b must be used to be able to reduce the test into a single tube [55]. The required temperature can be provided with a heat block or even a simple sous-vide cooker. RT-LAMP can be severely inhibited by saliva, thus requiring a prior RNA isolation step or a well-optimized step for the inactivation of salivary enzymes. Some COVID19 point-of-care diagnostic approaches use RT-LAMP such as COVID19 Penn-RAMP [84], HP-LAMP [85] and a colorimetric assay [86]. But it is suggested that using CRISPR-Cas12 improves the sensitivity and specificity of the tests compared to using only RT-LAMP [66].

Besides these amplification methods, there is a CRISPR-based approach that does not require nucleic acid amplification. It was previously reported that using different crRNAs together can increase the sensitivity of the test but Fozouni et al. demonstrated that multiple crRNAs can overcome the need for nucleic acid amplification [87]. They devised a protocol that can detect the SARS-CoV-2 genome quantitatively in pre-extracted RNA samples under 30 minutes. Test reached the sensitivity of 100 copies/μL.

Readout

There are mainly two approaches to detect amplicon in CRISPR-based COVID19 tests: Lateral flow assay and fluorophore quencher paired ssDNA/ssRNA. Lateral flow assay can be easily found anywhere as a paper dipstick, and it is very easy to apply. It can also be used in serological tests [88]. As it can be seen from Figure 1, there are two lines on the paper dipstick. The control line has streptavidin and if the FAM-biotin reporter is not cleaved, it is stuck in the control line due to biotin-streptavidin reaction. Whereas, if the Cas enzyme gets activated and cleaves the reporter, free FAM can reach the second line and be kept in place by antibodies specific for FAM. The important advantage of lateral flow assay over fluorophore signal is that it provides a simple, naked eye readout that does not require special instrumentation. It provides a simple qualitative result such as positive or negative. However, this qualitative result might be considered as a disadvantage as we will not be able to detect the viral titer and understand the condition of the patient. Opening the tube for lateral flow assay also brings a contamination risk. This contamination risk may prohibit its use outside a laboratory environment. Therefore, detection of fluorescence signals might be better in terms of contamination risk. A fluorescence readout could detect the viral load of patient samples in real-time. This quantitative information gives us the chance to observe the natural course of the disease and intervene accordingly. Detection of the fluorescence signal requires special instrumentation but it is demonstrated in many studies that a mobile phone can be used to detect the fluorescent signal [67,87]. Therefore, this requirement does not restrict its application as a point-of-care diagnostic test. Further, it is reported in a study that researchers received better signals (less background noise) with a mobile phone compared to the laboratory instrument [87]. The fluorescence signal can also be visualized with a blue LED and become observable without a device [66].

Cost

The cost of a point-of-care diagnostic test holds great importance since an expensive test has limited applicability. The cost of the RT-qPCR test may range from $25 to 100 per test and immunological tests cost around $6-8 per test [20]. Whereas, material costs of CRISPR-COVID were reported to be less than $3,5 on research scale [74]. It was previously reported that a SHERLOCK test can be redesigned and synthesized for as low as $0,61 per test [68]. Therefore, in terms of cost, CRISPR-based diagnostic tests are valuable candidates as point-of-care diagnostic tests.

Concluding Remarks and Future Perspective

CRISPR-based diagnostics have many advantages as emphasized throughout the article. They are very easy to perform and will become easier when these optimized reagents become commercially available. They do not require complex equipment or trained personnel. Required pieces of equipment are a pipette, a reaction tube, reagents, a heat block, and a mobile phone (or a paper dipstick). These types of equipment can be found anywhere. Therefore, it can be used widely in rural areas and at the bedside without referring to a central laboratory. Being independent of central laboratories and sample transportation would reduce the turnaround time substantially. Some of these methods even do not require RNA isolation and some are independent of nucleic acid amplification. This flexibility further decreases the sample-to-answer time (as can be seen in CRISPR-FDS). Obtaining such rapid results provide vital advantages in many cases. In schools, the students and the teachers could be tested before entering school. In airplanes, passengers could be tested before boarding. Therefore, these tests could prevent the spread of the virus more efficiently.

An ideal point of care diagnostic test is inexpensive, easy to perform, has short sample-to-answer time, and high accuracy. Among the reviewed CRISPR-based approaches, we found CRISPR-FDS to be the most suitable one as a point-of-care diagnostic test. Starting from the specimen collection, saliva samples can be collected by the patient himself. Therefore, reducing the extensive protective gear required for the healthcare worker. After specimen collection, instead of conventional RNA isolation, an optimized lysis protocol is followed. As a result, expensive reagents and commercial kits are not required for RNA isolation which reduces the cost and turnaround time substantially. The sample-to-answer time of CRISPR-FDS is only 15 minutes (5 minutes lysis and 10-minute RT-RPA amplification followed by a smartphone-based readout). Therefore, its sample-to-answer time occurs to be the shortest among the nucleic acid detection methods. The test yielded the limit of detection as 0,38 copies/µL, which is the second-best result after STOPCovid.v2. Swab samples of 103 patients that were tested with CRISPR-FDS

exhibited complete concordance with RT-qPCR results and saliva samples exhibited a 1,3% false-negative rate. Overall, we found CRISPR-FDS to be the best CRISPR-based approach for the diagnosis of COVID19 in terms of turnaround time, the limit of detection, sensitivity, and specificity. Other approaches also show promising results. In terms of clinical validation, SHERLOCK, STOPCovid, and DETECTR were the most extensively studied ones and they exhibited high sensitivity and specificity compared to RT-qPCR.

Recently, worrisome SARS-CoV-2 variants have emerged [89-91]. Currently available vaccines are thought to be effective against these variants [92-95]. However, a new variant can bypass the vaccination immunity and we should be prepared for such an event. CRISPR-based diagnostics share the same restrictions with RT-qPCR in terms of new variants. If there is a mutation in the primer binding sites or crRNA binding sites, both tests could fail to give accurate results. The important advantage of CRISPR-based diagnostics is that multiple different crRNAs can be efficiently combined and multiplexed [96]. That way, if there is a mutation in one of the crRNA binding sites, the difference between the obtained and expected fluorescence signal could alarm us when we face a new variant [87]. Next-generation sequencing will remain to be the golden standard for the identification of new variants/species [97] but CRISPR-based diagnostics might be helpful for screening. After the identification of a new variant, a specific crRNA could be easily designed and added to the protocol. As shown in the study of Fozouni et al., the combination of multiple crRNAs covered 4115/4118 of the genomic variants in the database [87]. The flexibility to design and use multiple crRNAs is regarded as one of the biggest advantages of CRISPR-based diagnostics.

To conclude, CRISPR-based diagnostics hold great potential as a next-generation point-of-care diagnostic test for COVID19, as well as many other infectious diseases like Zika virus, Dengue virus [68], Human Immunodeficiency Virus (HIV), and Mycobacterium Tuberculosis [53]. We have learned the importance of fast and accurate diagnosis during the pandemic and now we should focus on how to improve these tests further in terms of cost and widespread availability. RT-PCR's restrictions

are not just technical but also financial. Even though high-frequency testing with RT-PCR can help to control the spread of infectious disease, it has a significant economic burden on low-middle income countries. We have learned that worldwide precautions (instead of country-wide) are necessary to contain/prevent a pandemic. Every country should be prepared for novel variants/species and these tests may provide low-middle income countries a better ground to fight against infectious diseases. Hopefully, these tests may prevent another infectious disease or a new variant from becoming a pandemic.

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The idea for this article resulted from the Hacettepe University Phase 3 Class "A new era in the treatment of genetic disorders: RNA-dependent genome editing". In this class, we mainly discuss the features of the CRISPR-Cas system, its applications in basic and clinical research. The corresponding author Alp Uzay was one of the students taking the class and after his presentation on CRISPR-based COVID19 diagnostic approaches, it was decided to further investigate the subject.

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