

REVIEW



Potential of CRISPR/Cas system in the diagnosis of COVID-19 infection

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ABSTRACT

Introduction: Emerging novel infectious diseases and persistent pandemics with potential to destabilize normal life remain a public health concern for the whole world. The recent outbreak of pneumonia caused by Coronavirus infectious disease-2019 (COVID-19) resulted in high mortality due to a lack of effective drugs or vaccines. With a constantly increasing number of infections with mutated strains and deaths across the globe, rapid, affordable and specific detections with more accurate diagnosis and improved health treatments are needed to combat the spread of this novel pathogen COVID-19.

Areas covered: Researchers have started to utilize the recently invented clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (CRISPR/Cas)-based tools for the rapid detection of novel COVID-19. In this review, we summarize the potential of CRISPR/Cas system for the diagnosis and enablement of efficient control of COVID-19.

Expert opinion: Multiple groups have demonstrated the potential of utilizing CRISPR-based diagnosis tools for the detection of SARS-CoV-2. In coming months, we expect more novel and rapid CRISPR-based kits for mass detection of COVID-19-infected persons within a fraction of a second. Therefore, we believe science will conquer COVID-19 in the near future.

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1. Introduction

Coronavirus infectious disease-2019 (COVID-19) is caused by a severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). This COVID-19 first occurred in Wuhan, China, in late December 2019 [1,2]. Initially, the seriousness of COVID-19 was unknown. Later, this SARS-CoV-2 was identified to cause large-scale loss of life and severe human suffering in many countries. Since the pandemic of coronavirus (CoV) is increasing across the globe, scientists are focusing on developing the novel diagnostic tools, vaccines, and treatments. Some vaccines like Pfizer-BioNTech (BNT162b2), Moderna (mRNA-1273), Johnson & Johnson's Janssen (JNJ-78436735), Astra Zeneca and Oxford's Covishield, and Bharath Biotech's Covaxin have been approved for emergency use for SARS-CoV-2. However, the spread of the virus has not yet been stopped. New mutant variants such as B.1.1.7, B.1.1.7.2, B.1.526, and P.1 of the CoVs are creating serious health concern especially in India.

CoVs are a family of positive-sense, single-stranded RNA (ssRNA) viruses, which infect nose, throat, and sinuses leading to neurological and respiratory diseases in humans [3,4]. Prior to SARS-CoV-2, there were six others major CoVs species including SARS-CoV and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV) [4]. World Health Organization (WHO) declared that COVID-19 is a great infectious disease to the world [5]. Recent reports state that more than 161 countries are being affected severely due to COVID-19 [5,6]. Therefore, scientists are forced to develop efficient

vaccines to stop the spread of this novel SARS-CoV-2. At present, the SARS-CoV-2 infected persons are being isolated for 7–28 days, and severely infected patients are kept under supportive care [7,8].

Scientists are collecting the blood samples from the pneumonia patients and isolating the genomic sequence of SARS-CoV-2 to identify the mutants [9]. Based on the genomic characters, biopharmaceutical companies employ diagnosis tests namely qRT-PCR and reverse transcription loop-mediated isothermal amplification (RT-LAMP) to confirm SARS-CoV-2 [10–12]. However, these detection techniques suffer by providing false negative results, which allow the infected patients to continue the spread of the virus. It also requires sophisticated instruments and skilled manpower. Hence, an easy, simple, and quick diagnostic method with accuracy is essential for rapid diagnose of SARS-CoV-2 infection.

Scientists started to explore modern molecular tools including genome-editing systems, especially the recently invented and noble prize winning clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas) system for rapid diagnosis of SARS-CoV-2 [13,14]. CRISPR/Cas system has become a most popular genome-editing tool. This system uses a programmable protein that is attached to the targeted sequence with the help of guide RNA (gRNA) and creates double-stranded breaks (DSBs) [15]. There are many types of Cas proteins viz. Cas1, Cas2, Cas3, Cas6, Cas9, and Cas10 [16,17]. Among these, Cas9 protein is the efficient genome-editing system to target double-stranded DNA (dsDNA)

Article highlights

- We have highlighted the potential of the Nobel Prize-winning CRISPR/Cas system for the effective diagnosis of COVID-19 infections.
- SARS-CoV-2 and its newly evolving mutant variants such as B.1.1.7, B.1.526, P.1 of the CoVs are creating serious health concerns worldwide.
- With a constantly increasing number of infections and deaths across the globe, rapid, affordable, and specific detections with more accurate diagnosis and improved health treatments are needed to combat the spread of COVID-19.
- Presently RT-PCR testing method has been widely used as a highly specific detection method to detect the presence of SARS-CoV-2, but it has its own limitations.
- The CRISPR/ type II (Cas9), V (Cas12), and VI (Cas13)-based SHERLOCK, DETECTOR tools are projected for the rapid detection of novel COVID-19 are compared.
- We discuss the potential of the CRISPR/Cas system for the rapid diagnosis to enable efficient control of the COVID-19 infections.

or single-stranded DNA (ssDNA). Following Cas9 protein, the Cas12, and Cas13 proteins receive more attention in diagnosing viral diseases. Cas12 protein cleaves ssDNA and dsDNA, whereas Cas13 protein cleaves ssRNA; therefore, these proteins play an important role in the diagnosis of CoVs. In this review, we summarize the potential of CRISPR/Cas systems including Cas9, Cas12, and Cas13 proteins utilized for the diagnosis of SARS-CoV-2 infection.

2. SARS-CoV-2

The SARS-CoV-2 has a linear, and positive-stranded RNA which transmits deadly infections to all eukaryotic organisms [18]. It belongs to the families *Coronaviridae*, *Roniviridae*, and *Arteriviridae* and to the order *Nidovirales* [19,20]. They are classified into four genera: 1) Alpha coronaviruses that include human coronavirus NL63 (hCoV-NL63), porcine transmissible gastroenteritis coronavirus (PTGEV); 2) Beta coronaviruses that include SARS-CoV, coronavirus HKU4, MERS-CoV, bovine coronavirus (BCoV), bat, mouse hepatitis coronavirus (MHV) and human coronavirus OC43; 3) Gamma that include avian infectious bronchitis coronavirus (IBV); and 4) Delta coronaviruses that include porcine delta coronavirus (pdCoV) [21–23]. *Coronaviridae* and *Ronoviridae* have large-sized RNA viral genomes ranging from 26 to 32 kbp. It is covered by a helical capsid formed by nucleocapsid protein (N) [24] and surrounded by an envelope protein (E). The structure of viral associated proteins includes the membrane protein (M) [25] and E protein [26] that aid in virus assembly, and the spike protein (S) that allows the virus to enter into the host. Among these, the S protein is large which appears like a crown. Due to the presence of crown-like structure (latin word corona meaning crown), this virus is termed as coronavirus [18,27]

3. Diagnosis of SARS-CoV-2 infection

The SARS-CoV-2 pandemic has broken out widely all over the world. WHO informed that COVID-19 disease is pandemic and spreading rapidly, therefore urged for an efficient diagnostic methods. Scientists first isolated the unknown pathogen from

the pneumonia-affected person and did a metagenomic next-generation sequencing (mNGS) [28]. The novel CoV genome, which showed 79% similarity with the previously identified SARS-CoV [28,29]. Then CoV was diagnosed by the RT-PCR and chest computed tomography (CT) [30]. Readily available CT is used for detection in lung diseases, scientists used chest CT as an option to detect the CoVs [30]. Cheng et al. [31] tried to detect the novel CoV using chest CT [31]. They selected 8 CoV patients for CT imaging. They observed and reported that 63% patients had mild infections, 25% patients had moderate infections, and 13% had severe CoV symptoms. From these findings, they concluded that chest CT could provide early detection of CoV patients [31]. RT-PCR testing method was used as a highly specific messenger RNA detection method to detect the presence of CoV [31]. The RT-PCR test uses fluorescent dyes which enables the researchers to diagnose the suspects [31]. The Centers for Disease Control and Prevention from United States developed the primer which targets the viral *nucleocapsid* gene (N1 and N2), and WHO developed an assay that targets the virus RNA-dependent RNA polymerase gene and E gene [31]. However, RT-PCR requires excess reagents, skilled manpower and specialized equipment, which slows down the disease detection. Therefore, an advanced diagnostic method is needed to diagnose this emerging infection.

4. CRISPR/Cas system

CRISPR/Cas system is an RNA-guided endonuclease (RGEN)-based nucleic acid detecting tool that has changed the field of genomics, genome modification, gene therapy, and genome mapping [32,33]. The CRISPR and Cas proteins are adaptive immune systems against bacteria and archaea [34,35]. The CRISPR loci consist of a CRISPR array separated by a unique spacer sequence (30–40 bases). Adjacent to the CRISPR array, many Cas genes were discovered which encode the effector enzymes of the CRISPR system. The CRISPR-mediated immunity consists of three phases: (1) adaptation, (2) CRISPR RNA (crRNA) maturation, and (3) interference [36]. CRISPR/Cas system is divided into two classes based on the composition of the interference complex, namely Class 1 that includes types I, III, and IV, and Class 2 that includes types II, V, and VI [37,38]. Class 1 system utilizes crRNA with multi-effector complex to cleave target genome sequence, whereas the Class 2 system utilizes single multi-domain Cas protein with crRNA for interference (Table 1) [39]. In these classes, the type II CRISPR system has been widely adopted by the scientific community for more accurate and rapid diagnosis of infections [40–42]. Recently, CRISPR/Cas12 and Cas13 systems have been used in the diagnosis of human infections including bacteria and viruses [8].

4.1. CRISPR/Cas9 system

The type II CRISPR/Cas system is a bacterial adaptive immune system, consisting of Cas9 nucleases and the guide-RNA (gRNA) [45–47]. The CRISPR/Cas9 system creates blunt dsDNA breaks that are repaired by non-homologous end joining (NHEJ) or homologous recombination (HDR) methods. The

Table 1. Details on classification of CRISPR/Cas systems. The details such as class, type, protein name, target molecule, mechanism of spacer acquisition, processing of Pre-CRISPR, self vs non-self-discrimination, and host organism are included with their references.

Class	Type	Protein	Target	Spacer acquisition of CRISPR system	Interference of CRISPR system	Pre-CRISPR processing	Self vs non-self discrimination	Host organism	References
Class 1	I	Cas3	ssDNA	Cas1/Cas2/ Cas4	Cas7, Cas5, Cas8, and Cas3	Cas6	PAM	<i>E. coli</i>	[45]
	III	Cas10	ssDNA	Cas1/Cas2	Cas7, Cas5, and Cas1	Cas6	CRISPR Repeat	<i>S. epidermics</i>	[43]
	IV	Csf1	-	NA	Cas7, Cas5, and Csf1	-	-	-	[34]
Class 2	II	Cas9	dsDNA	Cas1/Cas2/ Cas4	Cas9	RNase III, and tracrRNA	PAM	<i>S. thermophilus</i> and <i>S. pyogenes</i>	[15,44]
	V	Cpf1	ssDNA and dsDNA	Cas1/Cas2/ Cas4	Cas12	Cpf1	PAM	<i>F. novicida</i>	[50]
	VI	C2c2	ssRNA	Cas1/Cas2	Cas13	-	-	-	[57]

PAM, protospacer adjacent motif; Cpf1, CRISPR-associated endonuclease in *Prevotella* and *Francisella* 1; tracrRNA, trans-activating CRISPR RNA, *E. coli*, *Escherichia coli*; *S. epidermics*, *streptococcus epidermics*; *F. novicida*, *Francisella novicida*

Cas9 nuclease isolated from *Streptococcus pyogenes* consists of two domains: (1) HNH endonuclease domain (single nuclease domain), and (2) RuvC endonuclease domain (three nuclease domains) [46,48]. The sgRNA which is derived from the fusion of crRNA and tracrRNA is complexed with the Cas9 nuclease targets the DNA through base pairing. This process targets the desired sequence with the help of protospacer adjacent motif (PAM)

4.2. CRISPR/Cas12 system

CRISPR/Cas12 is also known as CRISPR-associated endonuclease from *Prevotella* and *Francisella* 1 (*cpf1*); it belongs to the Class 2 type V associated nuclease [49,50]. CRISPR/Cas12 system is an efficient system that creates staggered cuts in both ssDNA and dsDNA. The Cas12 is derived from *Acidaminococcus* species Cas12a (*AsCas12a*) and *Lachnospiraceae bacterium* Cas12a (*LbCas12a*), which naturally evolves to fight against foreign genetic elements. The CRISPR/Cas12 system requires only the crRNA to create staggered cuts at the targeted sequence. The Cas12 nuclease contains two domains namely (1) nuclease lobe (NUC) domain and (2) RuvC domain for cleavage activity [51,52]. Like the Cas9 system, the Cas12 system targets the region besides the PAM region. Once Cas12 starts to encounter, it initiates R-loop formation which forms base-pair hybrids between the crRNA and the target sequence [53]. During this step, it matches the <17 bp target sequence and establishes an R-loop formation. Once R-loop is formed, the Cas12 nuclease uses its active RuvC domain and creates staggered cuts at the targeted ssDNA or dsDNA sequence with the presence of PAM sequence [50,54].

4.3. CRISPR/Cas13 system

CRISPR/Cas13 system is referred to as C2c2 system which belongs to the Class 2 type VI [55,56]. CRISPR/Cas13 system is another important system in the CRISPR world because it targets only ssRNA but not ssDNA or dsDNA. CRISPR/Cas13 system requires only crRNA to recognize the target [57]. The Cas13 consists of *alpha*-helical recognition (REC) lobe with a helical-1 domain and an NUC lobe with two higher eukaryotic

and prokaryotic nucleotide-binding protein (HEPN) domains [55,58]. These domains play a major role in RNA-targeted cleavage activity. Upon RNA binding, Cas13 protein gets activated by triggering the HEPN 1 domain to move the HEPN 2 domain, which will undergo a conformational change resulting in the cleavage of targeted ssRNA [59].

5. CRISPR/Cas9 system-mediated diagnosis of infectious diseases

CRISPR/Cas9 system has been adopted by the scientific community for the diagnosis of infectious diseases (Table 2). Pardee et al. [42] first demonstrated a simple novel method by combining CRISPR/Cas9 with isothermal amplification technique referred to as nucleic acid sequence-based amplification (NASBA) and detected Zika virus strain. The researchers utilized the dsDNA to intermediate the NASBA that served as a substrate for the Cas9 endonuclease. The sgRNA-Cas9 complex cleaves the resulting dsDNA, full-length strand (not truncated strands) activated the toehold switch sensors and led to color changes in order to identify different strains. From the result, investigators stated that this biomolecular platform resolved practical limitations and suggested that this could be used to develop molecular diagnostics for challenging the pandemic [42]. Guk et al. [41] developed a CRISPR/Cas9-mediated fluorescent *in situ* hybridization (DNA-FISH) method to detect methicillin-resistant *Staphylococcus aureus* (MRSA) [41]. In this method, the dCas9/sgRNA complex coupled with an SYBR green I fluorescent probe was used to target the *mecA* gene and recognize *mecA* gene associated with MRSA. The dCas9/sgRNA did not induce DNA cleavage but it recognizes the target DNA sequence, thus it is suitable for detection by FISH. The corresponding fluorescence was sufficient to efficiently detect the MRSA even at a lower concentration of 10 CFU/ml. From the result, researchers reported that dCas9/sgRNA-based detection is simple to detect various pathogens [41]. In another study, a combination of CRISPR/Cas9 with optical DNA mapping was employed to diagnose bacterial antibiotic-resistance genes [60]. In this demonstration, Cas9 and gRNA were fused that cleaved the targeted specific DNA sequences of resistance genes. Then fluorescent dye (YOYO-1)

Table 2. Details of CRISPR/Cas systems utilized for detecting pathogenic viruses. The details such as name of the CRISPR/Cas system, used, name of the effector, amplification method, targeted viruses, type of nucleic acids, specificity and detection time are included with their references.

CRISPR/Cas systems	System utilized	Effector	Amplification method	Target Virus	Type of Nucleic acid	Specificity	Detection time	References
CRISPR/Cas12	CRISPR/Cas12a	<i>LbCas12a</i>	RT-LAMP	SARS-CoV-2	ssRNA	High	<40 minutes	[61]
CRISPR/Cas13	SHERLOCK	<i>LwCas13a</i>	RT-RPA	Dengue Virus	ssRNA	High	5 hours	[78]
	SHERLOCKv2							
CRISPR/Cas13	POC	<i>LwCas13a</i>	RT-PCR	Ebola Virus	ssRNA	High	<2 hours	[62]
CRISPR/Cas13	SHERLOCK	<i>pspCas13a</i>	RT-RPA	Influenza Virus	ssRNA	High	<2 hours	[63]
	SHERLOCKv2							
CRISPR/Cas12/13	DETECTR	<i>LwCas13</i>	RT-RPA	White spot syndrome Virus	ssDNA and ssRNA	High	<1 hour	[64]
		<i>LbCas12a</i>	PCR					
CRISPR/Cas13	SHERLOCK	<i>pspCas13</i>	RT-RPA	VSV	ssRNA	High	<1 hour	[63]
CRISPR/Cas13	SHERLOCK	<i>LbCas13</i>	RT-RPA	PRSSV	ssRNA	High	3 hours	[65]
CRISPR/Cas13	HOLMES	<i>LbCas13</i>	RT-RPA	LCMV	ssRNA	High	2 hours	[63]
CRISPR/Cas12	HOLMES	<i>LbCas12a</i>	PCR	JEV	ssDNA	High	1 hour	[66]
CRISPR/Cas13	SHERLOCK	<i>LwCas13</i>	RT-RPA	EBV	ssRNA	High	1 hour	[67]
CRISPR/Cas13	SHERLOCK	<i>LwCas13</i>	RT-RPA	CPV-2	ssRNA	High	1 hour	[68]
CRISPR/Cas12	DETECTR	<i>LwCas13</i>	RT-RPA	ASFV	ssDNA	High	1 hour	[69]
CRISPR/Cas12	CRISPR-MTB-FISH	<i>Cas12a</i>	RPA	MRSA	ssDNA	High	1 hour	[41]
CRISPR/Cas9	Cas9	<i>SpdCas9</i>	PCR	M. Tuberculosis	dsDNA	High	<1 hour	[70]
CRISPR/Cas9	APC-Cas	<i>SpdCas9</i>	PCR	<i>Salmonella</i>	dsDNA	High	3 hours	[71]

SHERLOCK, specific high-sensitivity enzymatic reporter unlocking; DETECTR, DNA endonuclease-targeted CRISPR *trans*-reporter; POC, point of care; HOLMES, Hour Low-cost multipurpose highly efficient system; FISH, fluorescent *in situ* hybridization; RT-LAMP, reverse transcription loop-mediated isothermal amplification; RT-RPA, reverse transcription-recombinase polymerase isothermal amplification; PCR-polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; VSV, Vesicular stomatitis virus; PRSSV, Porcine reproductive and respiratory syndrome virus; LCMV, Lymphocytic choriomeningitis virus; JEV, Japanese encephalitis virus; H7N9, Avian influenza A virus; GRBV, Grapevine red blotch-associated virus; EBV, Epstein-Barr virus; CPV2, Canine parvovirus type 2 virus, ASFV, African swine fever virus; MRSA, Methicillin-resistant *Staphylococcus aureus*.

and netropsin bound to the resulting DNA based on AT-rich regions and detected each DNA segment of different resistant genes. The addition of multiple crRNA detected many different genes in each reaction. From these results, Muller et al. [60] concluded that CRISPR/Cas9 system in combination with optical DNA mapping could be applied for the detection of different pathogens (Figure 1).

Researchers detected dual genes of SARS-CoV-2 in a single test using the CRISPR/Cas9 system [72]. They first designed a triple-line lateral flow strip (TL-LFA) and later designed two sgRNAs (sgRNA1 and sgRNA2) for targeting *E* and *orf1ab*

genes with their scaffold sequences. The scaffold sequences are designed in such a way as having a binding site for employing AuNP-DNA probes. Then the sgRNAs were mixed with Cas9 and the complexes were run over the lateral flow strip, the AuNP-DNA probes bound to the scaffold sequence via DNA hybridization. Once AuNP accumulated, the colored bands are seen through the naked eyes that helps to detect the SARS-CoV-2. From these analyses, researchers confirmed that the CRISPR/Cas9 based TL-LFA strip assay would detect the SARS-CoV-2 accurately [72]. Recently, the 'CRISPR/Cas9 eraser' approach had been applied to detect the SARS-CoV-2

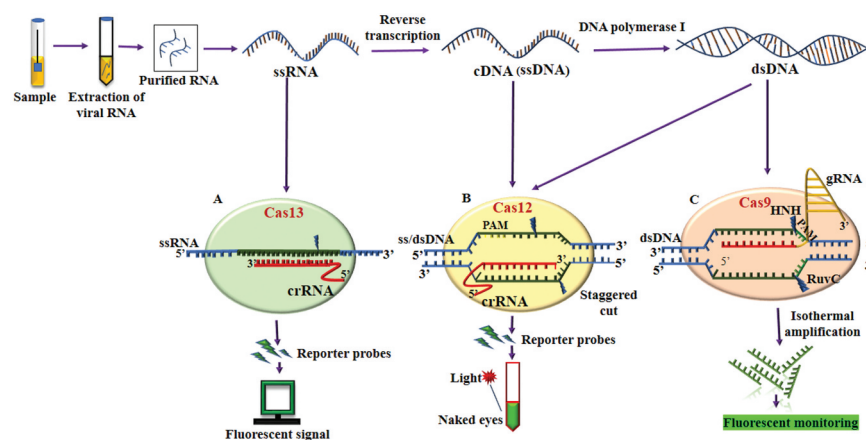


Figure 1. An overview of CRISPR/Cas system (Cas13, Cas12, and Cas9) based SARS-CoV-2 detection methods. Viral sample is collected from nasal, nasopharyngeal, and throat swabs of humans. The RNA is extracted from the virus and purified using specific RNA extraction kit. (A) The purified RNA is subjected to Cas13 system and using appropriate probe, the target is detected. (B) The purified RNA is subjected to reverse transcriptase (RT) and converted into complementary DNA (cDNA) and single-stranded DNA (ssDNA). The Cas12 system binds with the targeted sequence and discriminates its cleavage activity with the reporter probe and detects the virus. (C) The cDNA is converted into double-stranded DNA (dsDNA) using DNA polymerase I enzyme, which is recognized by the Cas9 system. It creates a double-stranded break (DSB) and upon cleavage, isothermal amplification is used to detect the targeted virus.

infection and swine flu virus [73]. CRISPR/Cas9 eraser was designed to accomplish a contamination-free RNA detection through RT-PCR. In addition, the researchers extended the CRISPR/Cas9 eraser to the PCR technique. They designed an artificial PAM site into the PCR products through primer designing and planned an approach named PAM-implanted PCR. They programmed conventional PCR primers by adding two extra cytosine (C) bases at the 3' end that leads to the amplified products containing the PAM site (artificial PAM site). The sgRNA which is corresponding to the amplified PCR products (identical artificial PAM site) was used for Cas9/sgRNA recognition and cleavage. Through CRISPR eraser, the researchers spotted the SARS-Cov-2 and swine flu virus with the suitable strip test [73].

6. CRISPR/Cas12 system-mediated diagnosis

CRISPR/Cas12 is a smaller system; it does not require tracrRNA, and cleaves dsDNA or ssDNA via the RuvC domain. Unlike Cas9, the Cas12 recognizes a different PAM sequence (5'-TTN-3'), upstream of its target. Researchers developed a different method called DNA endonuclease-targeted CRISPR *trans*-reporter (DETECTR) based on Cas12 system (Table 2) [8]. The DETECTR technique utilizes type V enzyme to directly cleave ssDNA sequence in three-stage process: (1) gRNA directs the Cas12 enzyme to ssDNA with targeted viral genome, (2) While binding to its targeted viral genome, a ssDNA binds with the quencher molecule and reporter fluorophore to cleave randomly by the Cas12 enzyme, (3) This collateral cleavage is detected by the fluorescent signal released from quencher and fluorophore [8]. This system showed the advantage of the diagnosis method to detect even a single molecule of viral particle (Figure 2). Chen et al. [8] first reported the DETECTR [8]. DETECTR technique achieved the attomolar sensitivity for DNA detection. In this work, they combined the nonspecific ssDNA of Cas12a and reported the rapid detection

of the virus. Utilizing DETECTR, they differentiated several human papillomaviruses (HPV16 and HPV18) from crude DNA extracts from clinical samples. The DETECTR technique identified HPV16 and HPV18 from clinical specimens and showed 25/25 and 23/25 results within 1 hour [8]. In addition, CRISPR/Cas12 was also used to diagnose the African Swine Fever Virus (ASFV) [74]. CRISPR/Cas12a was combined with fluorescent-based point of care (POC) system, which bound with and detected targeted ssDNA. Cas12/crRNA/ASFV DNA complex got activated upon binding and degraded the ssDNA. They detected the ASFV with a limitation of 1 pM within 2 hours. In addition, Cas12, crRNA and ASFV DNA complex continuously cleaved ssDNA complex, even after 24 hours, which displayed an improved detection limit of 100 femtomolar (fM). Cas12 system is very specific and can detect even up to a single nucleotide of targeted virus [74].

Researchers started to utilize the DETECTR system for the SARS-Cov-2 detection. The Mammoth Biosciences, Inc., San Francisco, CA, first diagnosed the SARS-Cov-2 by targeting the *N* and *E* genes through CRISPR/Cas12 system [9]. They engineered Cas12-gRNAs to specifically target the SARS-CoV-2 and optimized the DETECTR assay for *E* and *N* genes. They run this enhanced DETECTR assay and visualized the detection of SARS-CoV-2 on a lateral flow strip. From the analysis, a faster detection was observed through DETECTR, which saved more than an hour. The sensitivity range of the DETECTR platform was 70–300 copies/μl input [9]. The protocol encompassed LAMP in combination with reverse transcription instead of recombinase polymerase amplification (RPA)-based amplification that resulted in detection of SARS-CoV-2 within 30 min [9]. Similarly, CRISPR/Cas12-based fast and portable SARS-CoV-2 detection was employed by Argentina and CASPR Biotech [75]. They collected saliva specimens from COVID-19 patients and reported that naturally occurring molecules present in saliva did not influence any inhibitory problems during the CRISPR/Cas12-based paper strip assay [75].

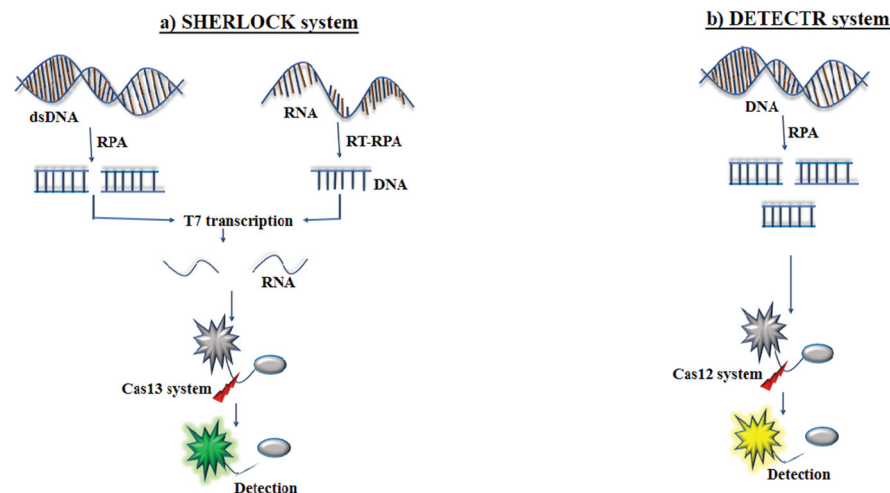


Figure 2. Mechanism of SHERLOCK and DETECTR system. (A) Targeted double-stranded DNA (dsDNA) or RNA is amplified with recombinase polymerase amplification (RPA) or reverse transcription (RT)-RPA. The RPA is coupled with T7 transcription to convert targeted RNA for detection by Cas13 system. This amplification steps in combination with the reporter probe enables specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) to detect the targeted sequence, (B) In DNA endonuclease-targeted CRISPR *trans* reporter (DETECTR), DNA is amplified with RPA. The Cas12 system pairs with the single-stranded DNA (ssDNA) of interest, the DNase activity of Cas12 system is initiated. This amplification step in combination with the reporter probe enables DETECTR to detect the targeted sequence.

Additionally, a cross-institution from China checked an unambiguous detection of SARS-CoV-2 using the CRISPR/Cas12 system [76]. They designed crRNAs targeting *orf1a*, *orf1b*, *N*, and *E* genes of SARS and its related virus (Wuhan-Hu-1 strain; GenBank. No: MN908947), which detected the single nucleotide polymorphisms (SNPs). Based on the sensitivity and fluorescence intensity, *E*-crRNAmix was employed in screening SARS-CoV-2, whereas other target genes such as *orf1a*-crRNAmix, *orf1b*-crRNAmix, and *N*-crRNAmix also additionally confirmed the diagnosis of virus [76]. From the results, Feng and Li stated that CRISPR/Cas12-based detection could be utilized for the efficient and rapid diagnosis of COVID-19 (Figure 1) [76]. Recently, Jiang et al. [77] detected SARS-CoV-2 by developing a magnetic-pull-down-assisted colorimetric method associated with CRISPR/Cas12 system (M-CDC). For this method, they used gold nanoparticle (AuNP) probes to detect SARS-CoV-2. In addition, they screened 41 viral samples using M-CDC and observed performance of more than 95%, which is equal to the approved RT-PCR technique for detecting the virus. Thus M-CDC seems to be an efficient method for screening the SARS-CoV-2 without the need of sophisticated instruments [77].

7. CRISPR/Cas13 system-mediated diagnosis

The CRISPR/Cas13 mediated genome-editing is also utilized for a highly sensitive pathogen-detection (Table 3) [55,78]. The first reported platform of CRISPR/Cas13 based system is called as specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) (Figure 2) [79]. SHERLOCK technique combines isothermal and RPA or reverse transcription (RT)-RPA with nuclease activity of Cas13. The crRNA-Cas13 complex attached to the target nucleic acids cleaves the targeted nucleotides as well non-targeted RNA which are coupled with a fluorescent reporter that provides accurate fluorescent signal for real-time detection of pathogens. Gootenberg et al. [79] diagnosed *E. coli* and *Pseudomonas aeruginosa* with the DNA isolated from these strains using SHERLOCK system. They have also discriminated the isolates of *Klebsiella pneumoniae* with two types of resistance genes such as *K. pneumoniae* carbapenemase (KPC) and New Delhi metallo- β -lactamase 1. In addition, SHERLOCK allowed the discrimination of the targets that differ in only a single base-pair and distinguished successfully as African and American strains of ZIKA virus, serotype of dengue virus (DENV), cancer-related DNA target, and three ssRNA targets in a single reaction [79]. Gootenberg et al. [79] reported that this SHERLOCK could be used to detect targeted nucleic acids with higher sensitivity within 30 minutes including Zika virus and DENV. SHERLOCK has also been applied for the viral diagnosis [79]. Researchers developed a novel method by binding SHERLOCK with heating unextracted diagnostic samples to obliterate nucleases (HUDSON) for the detection of viral infection [80]. This method detects the DENV in the saliva, blood, and serum of viral infected patient within 2 hours [80]. Such platforms paved the way to utilize this technique to battle against COVID-19 or any other infectious diseases. In 2020, SHERLOCK Biosciences, Inc. and

Mammoth Biosciences, Inc. brought out a new combination for diagnostics of COVID-19 by utilizing SHERLOCK technique. They first amplified the viral RNA using RPA and performed *in vitro* transcription of amplified DNA into RNA. They designed sgRNA targeting *S* and *Orf1ab* genes and detected RNA using Cas13 nuclease. Then they used a commercially available paper stick for visual color readout and captures the cleaved reporter RNA. From the readout results, the researchers informed that this protocol would detect the SARS-CoV-2 target RNA sequence with a higher sensitivity of 10 to 100 copies/ μ l. The Mammoth Biosciences, Inc. stated that the SHERLOCK technique would detect the SARS-CoV-2 within an hour without any special instrumentation. Metsky et al. [81] evaluated the SARS-CoV-2 assay by utilizing CRISPR/Cas13 detection system [81]. The researchers created a website for CRISPR/Cas13 based assay designs to detect 67 viral species including the SARS-CoV-2. Using assay designs they screened the 4 SARS-CoV-2 designs with the help of CRISPR/Cas13 system. They incorporated synthetic RNA targets with fluorescent visualizers and detected 10 copies/ μ l of synthetic SARS-CoV-2-RNA [81].

Rauch et al. [82] applied Cas13-based-Ragged-Equitable-Scalable Testing (CREST) protocol to check the potency to detect SARS-CoV-2 [82]. This system involves the use of simple, effortless to operate, portable, and plastic-filter-based LED visualizers. In previously published works, researchers detected target RNAs using Cas13 nuclease with the help of lateral flow immunochromatography strips, but these strips demand a high cost. Therefore, they designed cost less P51 cardboard fluorescence visualizer (using 9 V battery) instead of immunochromatography strips and detected 10 copies of a target RNA molecule/ml. In addition, they used the smartphone camera for documenting results, and consequent uploading to the cloud to facilitate POC testing [82]. It advances the CRISPR based nucleic acid detection and shows its potency to shift on diagnostic and surveillance endeavors through direct screening even at a large group of populations [82]. Later, Tian et al. [83] group introduced the CRISPR/Cas13a assay for single-molecule RNA diagnosis which eliminated the method of nucleic acid extension and RT procedure. They reported that the CRISPR/Cas13a assay would be a powerful tool to detect the SARS-CoV-2 [83]. From this demonstration, they conclude that the CRISPR/Cas13 system could easily support both patients and public health practitioners for efficiently detecting the SARS-CoV-2 (Figure 1).

8. Economic feasibility of CRISPR/Cas system-based diagnosis

As an emergency situation for diagnosing the SARS-CoV-2, many industries started to discover economically viable kits by utilizing CRISPR/Cas system. As a result, they used DETECTR and SHERLOCK systems for rapid and accurate diagnosing of SARS-CoV-2 [84]. The Mammoth and Biosciences, Inc already started selling the DETECTR and their diagnostic kits in the market to detect viral and bacterial infections [85]. Therefore, the DETECTR and SHERLOCK methods play a major role in

Table 3. Details on molecular diagnostic tools-based detection tests used for SARS-CoV-2. The details such as test name, type of test, source of sample, targeted genes, results, test time and name of the organization with their country are included.

Test Name	Type of test	Sample source	Targeted genes	Test time	Organization Name	Country
iAMP COVID-19 detection Kit	RT-RPA	Nasal, nasopharyngeal and throat swabs	<i>ORF1</i> and <i>N</i>	<1.5 hours	Atila Biosystems Ltd.	United States
ID NOW COVID-19	RT-RPA	Nasal, nasopharyngeal, and throat swabs	<i>RdRP</i>	<5 minutes	Abbott Diagnostics Scarborough Ltd.	United States
SARS-CoV-2	RT-PCR	Nasal, nasopharyngeal, throat swabs, midturbinate nasal swabs and bronchoalveolar lavage fluid	<i>N1</i> and <i>RdRP</i>	<1 hour	Diagnostic Molecular Laboratory	United States
RT-PCR (COVID-19)	qRT-PCR	Nasal, nasopharyngeal, throat swabs, midturbinate nasal swabs, bronchoalveolar lavage fluid, aspirates, and sputum	<i>N</i>	4 days	LabCorp Laboratory Corporation of America	United States
ARIES SARS-CoV-2 assay	qRT-PCR	Nasal	<i>ORF1</i> lab and <i>N</i>	2 hours	Luminex Corporation	United States
SARS-CoV-2	RT-RPA	Respiratory samples	<i>E</i> and <i>N</i>	30–40 minutes	Mammoth Biosciences	United States
Accula SARS-CoV-2	PCR	Throat and nasal swabs	<i>N</i>	30 minutes	Mesa Biotech Inc.	United States
QIAstat-Dx Respiratory SARS-CoV-2	qRT-PCR	Nasopharyngeal	Viral RNA	1 hour	Qiagen GmbH	United States
TaqPath COVID-19 combo Kit	qRT-PCR	Oropharyngeal, nasopharyngeal, and anterior nasal	<i>ORF1</i> , <i>S</i> , and <i>N</i>	-	Rutgers Clinical Genomics Laboratory	United States
cobas SARS-CoV-2	qRT-PCR	Nasal and nasopharyngeal	Viral RNA	5 hours	Roche Molecular Systems. Ltd	United States
Viracor SARS-CoV-2 assay	qRT-PCR	Oropharyngeal, nasopharyngeal, and anterior nasal	<i>N</i>	18 hours	Viracor Eurofins Clinical Diagnostics	United States
BioFire COVID-19	qRT-PCR	Nasopharyngeal	<i>ORF1ab</i> and <i>ORF8</i>	1.5 hours	bioFire Defense LLC.	United States
CDC-2019	PCR	Oropharyngeal, Nasopharyngeal, and anterior nasal, nasal swabs, bronchoalveolar lavage fluid, aspirates and sputum	<i>N</i>	-	CDC-US	United States
Xpert Xpress SARS-CoV-2	PCR	Nasal and nasopharyngeal	<i>N2</i> and <i>E</i>	45 Minutes	Cepheid	Australia, Canada, Singapore, and United States
Panther Fusion SARS-CoV-2	PCR	Nasopharyngeal	<i>ORF1 lab 1</i> and <i>2</i>	<3 hours	Hologic Inc.	Australia and United States
MIRXES FORTITUDE KIT	qRT-PCR	Nasopharyngeal	Viral RNA	90 Minutes	mIREXES Pte Ltd.	Singapore
VitaPCR SARS-CoV-2	PCR	Nasal and nasopharyngeal	Viral RNA	20 Minutes	Credo Diagnostics Biomedical Ptd. Ltd.	Singapore
Lyra SARS-CoV-2	qRT-PCR	Oropharyngeal and nasopharyngeal	<i>Pp1ab</i>	75 Minutes	Diagnostics Hybrid Inc.	Canada
Nucleic Acid Diagnostic Kit (COVID-19)	qRT-PCR	Oropharyngeal, nasopharyngeal, serum, blood, and fecus	<i>ORF1 lab 1</i> and <i>E</i>	30 Minutes	Sansure Biotech Inc.	China
Standard M nCoV RT Detection Kit	qRT-PCR	Oropharyngeal swabs	<i>E</i> and <i>RrRP1</i>	50 Minutes	SD Biosensor	South Korea

RT-RPA, reverse transcription-recombinase-polymerase isothermal amplification; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; *N*, nucleocapsid; *ORF1*, open reading frame; *E*, envelope; *pp1ab*, poly proteins.

detecting SARS-CoV-2 within an hour or less with cost-effective detection. The other companies like Sigma-Aldrich also provide commercial CRISPR/Cas9 kits which detects bacterial and viral infections (Table 3). Recently, DIY scientists' company offered CRISPR kits for individuals at a low cost of 200 USD. Hopefully, these CRISPR/Cas-based techniques will offer cheap and rapid diagnostics option for other novel pathogens in future.

9. Potential of CRISPR/Cas system in SARS-CoV-2 diagnosis

CRISPR/Cas system has a great potential in various disciplines such as basic sciences, drug development, and developing molecular diagnostics against novel pathogens like SARS-CoV-2. The CRISPR/Cas systems could be a simpler tool to detect SARS-CoV-2 because these tools have successfully detected other pathogens with higher efficiency within 30–60 minutes. As already known, the recently invented CRISPR/Cas12/Cas13 systems cleave both ssDNA and ssRNA. Therefore, several researchers started to utilize the CRISPR/Cas systems for detecting pathogens and discovered fluorescent-labeled ssDNA/RNA probe for developing DNA-based diagnostic tool [86]. SHERLOCK technology uses the CRISPR/Cas13 system to identify the RNA and breaks collateral RNA along with targeted RNA. The non-targeted RNA is tagged to a fluorescent dye for identifying the targeted RNA. Zhang et al. [86] used CRISPR/Cas13 system-based SHERLOCK technique by utilizing two crRNAs which identify the signatures of SARS-CoV-2 [86]. Recently, US FDA approved SHERLOCK technology as an emergency use authorization (EUA) for the detection of SARS-Cov-2 [9]. Ding et al. [87] introduced All-In-One Dual CRISPR/Cas12 (AIOD-CRISPR) assay as an ultra-sensitive and faster assay to detect the target nucleic acid [87]. This AIOD-CRISPR assay mixtures are incubated at 37°C to prevent contaminations, which simplifies the detection procedure. Utilizing AIOD-CRISPR assay, they detected the RNA of HIV and SARS-Cov-2 within 60 minutes [87].

These overall studies proved the potential of the CRISPR/Cas system as an effective diagnostic tool for SARS-Cov-2 infections. Now we see only the beginning of the CRISPR based system for the diagnosis of SARS-CoV-2. Without a doubt, the potential of the CRISPR/Cas system can soon become the leading diagnostic tool for SARS-CoV-2 infection including the mutant strains.

10. Pros and cons of CRISPR/Cas-based diagnosis for infectious disease

In the modern era, many advanced diagnostic methods have been employed to diagnose and combat viral infections. The sudden emergency of novel COVID-19 virus has changed the life-style of humans. The major hurdles are the lack of proper equipments, time-consuming disease diagnosis methods, lack of low-cost techniques for COVID-19 diagnosis, and applying the diagnostic technique in remote areas (higher risk of disease transmission) especially in the developing countries. COVID-19 causes two types of infection: (1) symptomatic and (2) asymptomatic (causes a higher risk of getting unnoticed infectious disease transmission). Therefore, the availability of

rapid, accurate diagnostic tools may help in screening the infected individuals and stop the chain of disease transmission by SARS-CoV-2 virus.

Recently invented CRISPR/Cas system plays a vital role in developing novel molecular diagnostic tools to detect SARS-CoV-2. The sensitive detection of COVID-19 infection by using CRISPR/Cas-based SHERLOCK, DETECTR, HOLMES and SHERLOCKv2 are good methods for rapid diagnosis and control of SARS-CoV-2 [35,88]. The CRISPR/Cas-based SHERLOCK and DETECTR techniques distinguish SNP, HPV, Zika, and DENV strains [88].

CRISPR-based diagnostic tools provide a great advantage than the currently used serologic tests, nucleic acid associated tests, and RT-PCR tests for the diagnosis of SARS-CoV-2. The major advantage of the CRISPR system is, it does not require any expensive experiments, highly sensitive, and large-scale screening of infected peoples can be detected within a few minutes that results in stopping the community spread of the SARS-CoV-2 and leads to reduce morbidity and mortality. Even though the standard diagnosis RT-PCR is effective and sensitive, it requires sophisticated instruments, expert manpower, and cost. Therefore, CRISPR-based detection fulfills all the requirements like being easy to use, fast, and applicable in all portable molecular labs. However, the demerits of the CRISPR/Cas system should also be validated before introducing in diagnosing the SARS-CoV-2. The disadvantage is an 'off-target' effect which results in false results even though multiple online software tools are available to reduce the off-target effects. Another major disadvantage is the Cas proteins utilized by the CRISPR system which have nucleotide mismatches tolerance that causes nonspecific binding to the targeted template sequences which results in false detection of the target. Also, apart from being a lab based and more speculative detection method, CRISPR-based tests may take more time to make it as a rapid and domestic strip-based test which are normally used for pregnancy tests for the diagnosis of SARS-CoV-2 virus.

Improvement in these techniques would detect the viral infection even faster. Recently, a CRISPR chip has been discovered that detects the virus within 15 min [89,90]. However, developing the new CRISPR/Cas-based diagnostic methods must be carefully validated and tested including off-target phenomenon before functioning, because it may lead to poor signaling and a mistake in the results.

11. Conclusions

The limitations in traditional diagnosis tools open novel ways to utilize recent molecular diagnostic tools for detecting pathogens very quickly and efficiently. CRISPR type II (Cas9), V (Cas12), and VI (Cas13) systems brought an advanced diagnostic method for rapid detection and control of the pathogens including SARS-CoV-2. In the near future, we can also expect a mobile-powered CRISPR/Cas-based alarm system that will provide an early warning if any viral infected patient is nearby. However, inventing the novel CRISPR-based diagnosis tools requires speed and accurate detection. In addition, the evaluation of risks correlated with the utilization of the CRISPR/Cas system for diagnosis of infectious disease will be an important risk factor for the future.

12. Expert opinion

Outbreak of the global pandemic COVID-19 due to SARS-CoV-2 has threatened the entire world. Rapid spread of further mutated SARS-CoV-2 thus caused severe health and economic burdens that require us to develop advanced and ideal strategies for the quick diagnosis and treatment of COVID-19 patients. Recently, SARS-CoV-2 infectious disease has moved to an alarming phase of the second wave in many countries especially India which witnessed a rapid increase of the infected cases and deaths. The spread and death rates are more severe during March–May 2021 in India compared to other countries. The major hidden threat of the COVID-19 disease is asymptomatic carriers. Increasing the frequency of mass-screening of the population is the only and primary strategy to identify and isolate the individuals from spreading the virus. It is really challenging especially in the countries like India. Utilizing specific diagnostic tools of SARS-CoV-2 with quick intervention is believed to check and control the spread of the infectious disease to others.

For an early and efficient detection of SARS-CoV-2, currently the RT-PCR test is being used. However, studies revealed that utilizing RT-PCR techniques to diagnose SARS-CoV-2 demands a longer time to get the results. Furthermore, RT-PCR results sometimes fails to recognize the infected patients as it depends on capturing and detecting the virus that does not deliver any additional evidence on other symptoms. Delaying the RT-PCR test of the collected upper and lower respiratory track samples decreased the viral dosage in the sample, which leads to the negative results of the samples collected. Therefore, combination of RT-PCR with X-ray, and antigen test results are required to stop the chain of COVID-19 transmission among individuals.

While WHO reported about the spread of SARS-CoV-2 infection all over the world, the CRISPR researchers without the second opinion, started to explore the CRISPR systems against the diagnosis of SARS-CoV-2 infection. Since the CRISPR system plays an effective role in defending against viral infection, scientists utilized it as a novel natural diagnostic tool against SARS-CoV-2 for human benefit. In this review, we have highlighted the potential of the Nobel Prize winning CRISPR/Cas system in detail for the effective diagnosis of SARS-CoV-2 infections. This review article covered all the studies on CRISPR/Cas system such as CRISPR/Cas9/12/13 systems till date with their methods for diagnosing the SARS-CoV-2 infection. Therefore, this article is timely and interesting that may help to develop more novel, rapid diagnostic tools based on CRISPR/Cas systems for the treatment of SARS-CoV-2 infection.

Recently, CRISPR/Cas-based SHERLOCK diagnostic tool has been successfully approved by the US-FDA as an efficient diagnostic tool to detect SARS-CoV-2 infection. This approval has encouraged CRISPR researchers to find many novel diagnosis kits based on the CRISPR system. After SHERLOCK, the CRISPR/Cas-based DETECTR system joins the list in diagnosing the SARS-CoV-2. DETECTR system takes less than 15 minutes to detect SARS-CoV-2. Multiple groups are actively involved in developing diagnostic kits utilizing CRISPR-based tools. However, all these techniques should come into the marker for diagnosing SARS-CoV-2 infections at a low cost before the outburst of a deadly third wave throughout the world.

It will not be surprising if we expect more CRISPR-based kits in the coming months for novel and rapid tests for mass detection of SARS-CoV-2 infected persons within a fraction of seconds. This may help to detect the infected persons more efficiently with portable detection kits in rural parts of developing countries like India where testing rate is limited due to lack of skilled manpower and RT-PCR instruments. Science has overcome many pandemics and diseases like polio, smallpox, chicken pox, hepatitis, malaria, etc. by prudent application of diagnostic tools and treatments. The best example is the eradication of polio by several countries. Similarly, science will conquer COVID-19 in the near future and CRISPR/Cas system may play a key role in the efficient diagnosis and treatment of this mass-spreading disease.

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