Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

CHAPTER

CRISPR use in diagnosis and therapy for COVID-19

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Pallavi Deol[a], Aashwina Madhwal[b], Gaurav Sharma[a], Rahul Kaushik[c], and Yashpal Singh Malik[d,*]

[a]*Virology Lab, Centre for Animal Disease Research and Diagnosis, ICAR-Indian Veterinary Research Institute, Bareilly, India*

[b]*Division of Pathology, ICAR-Indian Veterinary Research Institute, Bareilly, India*

[c]*Laboratory for Structural Bioinformatics, Center for Biosystems Dynamics Research, RIKEN, Yokohama, Japan*

[d]*College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India*

**Corresponding author: e-mail address: malikyps@gmail.com*

Abbreviations

AAV	adeno-associated vectors
ACE-2	angiotensin converting enzyme-2
AIOD-CRISPR	All-In-One Dual CRISPR-Cas12a
CARVER	Cas13-assisted restriction of viral expression and readout
Cas	CRISPR associated
CASdetect	CRISPR-assisted detection
CONAN	Cas3-operated nucleic acid detection
COVID-19	Coronavirus Disease 2019
CREST	Cas13-based, Rugged, Equitable, Scalable Testing
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
crRNAs	CRISPR RNAs
DETECTR	DNA Endonuclease-Targeted CRISPR Trans Reporter
DR	direct repeats
EUA	emergency use authorization
FELUDA	FnCas9 Editor Linked Uniform Detection Assay
GFP	green fluorescent protein
gRNA	guide RNA
HEDGES	High-level Extended Duration Gene Expression System
HEPN	higher eukaryotes and prokaryotes nucleotide binding domains
HPV	human papilloma virus
IAV	influenza A virus

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ICTV	International Committee on taxonomy of viruses
iSCAN	*in vitro* Specific CRISPR-based Assay for Nucleic acids detection
LAMP	loop-mediated isothermal amplification
LCMV	lymphocytic choriomeningitis virus
LFA	lateral flow assay
LoD	limit of detection
LSPCF	Localized Surface Plasmon Coupled Fluorescence
LSPR	localized surface plasmon resonance
NER	naked eye readout
NP	nasopharyngeal
ORF	open reading frame
PAC-MAN	prophylactic antiviral CRISPR in human cells
PAM	protospacer adjacent motif
PFS	protospacer flanking sequence
POC	point-of-care
PRRSV	porcine reproductive and respiratory syndrome virus
RBD	receptor binding domain
RdRP	RNA-dependent RNA polymerase
RNP	ribonucleoproteins
RPA	recombinase polymerase amplification
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus- 2
SHERLOCK	Specific High-Sensitivity Enzymatic Reporter Unlocking
SHINE	SHERLOCK and HUDSON Integration to Navigate Epidemics
SNP	single nucleotide polymorphism
STOP	SHERLOCK Testing in One Pot
tracrRNA	trans-activating CRISPR RNA
UTM	Universal Transport Media
VaNGuard	Variant Nucleotide Guard
VSV	vesicular stomatitis virus

1 Introduction

An outbreak of a new human respiratory disease was noticed in late December 2019 at Wuhan city, Hubei province, China, caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), a new emerging corona virus (Jiang et al., 2020). It has now infected over 211 million people worldwide with over 4.4 million deaths since its onset (https://covid19.who.int/; accessed on August 24, 2021). The Global Initiative on Sharing Avian Influenza Data (GISAID) website published the first genome sequence of SARS-CoV-2 on January 10, 2020 and since then a plethora of sequences have been released through the GISAID platform (Hu, Guo, Zhou, & Shi, 2021). On January 9, 2020, the etiological agent was identified as a never seen before *betacoronavirus*. Studies claimed that the 2019-nCoV (novel corona virus) was found to be 96% identical at the whole-genome level to a bat coronavirus (Zhou et al., 2020). On February 11, 2020, the International Committee on taxonomy

of viruses (ICTV) named the virus 2019-nCoV SARS-CoV-2 and the World Health Organization (WHO) named the disease coronavirus disease 2019 (COVID-19), then declared it as a pandemic on March 11, 2020 (https://www.who.int/emergencies/diseases/novelcoronavirus2019). The whole of the globe was threatened causing high morbidity and significant mortality. The SARS-CoV-2 virus belongs to the family of enveloped positive-sense RNA genome viruses that infects both the upper and lower respiratory tracts (Lu et al., 2020; Malik et al., 2021). The virus genome is made up of 06 functional ORFs, arranged (5′ to 3′) as ORF1a/1b (replicase; covers 2/3rd of the 5′ genome and encodes for polyprotein 1ab), spike surface glycoprotein (S), small envelop protein (E), membrane protein (M), and nucleocapsid (N). It also includes an RNA-dependent RNA polymerase (RdRP) which maintains genome fidelity (Malik et al., 2021; Sexton et al., 2016).

Among structural proteins, the receptor binding spike surface glycoprotein, which enables the virus to infect cells, is encoded by the "S gene." In terms of nucleotide sequence-based analysis, the "S-gene" of SARS-CoV-2 was observed to be phylogenetically divergent from its previously known counterparts in other corona viruses (Malik, Kumar, et al., 2020; Malik, Sircar, et al., 2020; Udugama et al., 2020). However, the receptors used by SARS-CoV-2 are similar to the previously known SARS-CoV, *i.e.,* angiotensin converting enzyme-2 (ACE-2) (Zhou et al., 2020). Some of the common symptoms of COVID-19 includes throat pain, fever, body pain, cough and cold at initial stages of infection. In severe cases there may be weakness, shortness of breath, skin rashes and congestion of conjunctiva. Certain cases also revealed difficulty in breathing leading to severe hypoxia causing death (Malik, Kumar, et al., 2020; Malik, Sircar, et al., 2020).

2 Diagnostics and therapeutics for SARS-CoV-2

Rationally, emergence of any pandemic accelerates researchers to develop rapid, accurate, and ultrasensitive disease detection kits enabling the rapid implementation of control measures. As COVID-19 shows both symptomatic and asymptomatic traits, its early diagnosis remains vital for pandemic control and establishment of an adequate therapeutic strategy for reducing the disease threat (Pizzol et al., 2020). The basic approach of diagnosis starts from clinical symptoms ruling out the aetiology leading to a cause confirmed by laboratory diagnosis. The designing of a better technique completely relies on (Abbott et al., 2020) the proteomic and genomic composition of the pathogen and/or (Abudayyeh et al., 2017) changes in the expression of genes (proteins) in the host during and after infection (Udugama et al., 2020). This is based on two approaches (1) Immunological (*viz*. RAPID, ELISA, LFT) and 2) Molecular (*viz*. qPCR, LAMP). Between both, immunological approaches involve serological tests to detect the antigens (structural protein of virus) in lungs or antibodies in blood. It offers an enhanced understanding of the ongoing mechanism and the dynamics of disease transmission (Mahase, 2020). Whereas, the molecular approaches include mainly nucleic acid detection by

different methods which helps in early diagnosis of the disease. A list of other adjunct diagnostic techniques are also exploited/developed to identify the disease including non-invasive techniques like ultrasound as point-of-care ultrasound (POCUS), CT scan, piezoelectric biosensing (surface of piezoelectric crystals are bounded by SARS-CoV2 horse polyclonal antibodies from protein A), gold nanoparticles biosensing (exploited largely for MERS CoV and HCoV viruses), LSPR (Localized surface plasmon resonance approach), and optic immunosensors (LSPCF) (Layqah & Eissa, 2019). The recorded consistent lung sonographic findings in a case series of 20 patients in China with confirmed COVID-19 revealed pleural line irregularity and thickening, focal B-lines, bilateral diffuse B-profile with spared areas, subpleural consolidation and, rare pleural effusion (Peng, Wang, & Zhang, 2020; Poggiali et al., 2020).

Among all the diagnostic techniques, PCR is one of the most adopted procedures for detecting viral nucleic acids, and it has been declared as the gold standard approach to diagnose the viral infections because of high sensitivity and accuracy. Though, the quantitative PCR (qPCR) may be performed to diagnose COVID-19, restricted availability to RT-qPCR equipment and materials may cause the diagnostic procedure to take longer and to further complicate the situation because lower viral loads may go undetected, resulting in false negative results (Broughton et al., 2020; Wang, Doyle, & Mark, 1989). Hence, there is an immediate need to adopt a diagnostic method with simplistic, time-efficient and highly accurate throughput for diagnosing the emerging pathogen in the early stages of infection. Most recent molecular diagnostics that can detect the presence of infection despite lower viral titres can be beneficial to ensure timely diagnosis of all infected patients (Xiang et al., 2020).

A quick detection of the virus serves half the game. However, successful therapeutic interventions are equally necessary to combat a viral disease. The development of virus specific therapies is a daunting task owing to the involvement of host factors in viral life cycle, due to which the number of approved antiviral therapies are limited (De Clercq & Li, 2016). The mainstream therapeutic approaches for the current pandemic of COVID-19 includes preventing the SARS-CoV-2 virus from multiplication, which can be achieved by the use of the antiviral drugs (anticipated benefits early in the course of disease) and/or with immune modulators, which can modulate the immune response thereby helping the immune system fight against the virus (more effective in the later stages of COVID-19) (https://www.covid19treatmentguidelines.nih.gov/therapeutic-management/). Several hundred antivirals, immunomodulators, neutralizing antibody therapies *etc*. are being analysed to discover effective treatments for the COVID-19 (https://www.raps.org/news-and-articles/news-articles/2020/3/covid-19-therapeutics-tracker). Due to the immediate needs, the repurposing of some of the existing antiviral drugs is also seen as a feasible option. Along with the available options, it is equally important to investigate the diversified diagnostic and therapeutic approaches. Research is underway to develop novel rapid diagnostic techniques and to understand the effect of distinct categories of potential treatments against SARS-CoV-2.

In the last few decades, genetic engineering techniques have immensely improved the concept of disease diagnostics and therapeutics. CRISPR biotechnologies, where the Cas proteins act as effectors to recognize and degrade specific genome targets, complimentary to a specific guide RNA (primarily a tool for genome editing), is emerging as a potential tool for the development of new generation diagnostics, prophylactics as well as therapeutics. In recent years, research has explored the potential of different CRISPR/Cas systems as a tool for the development of novel diagnostics and therapeutics, due to specificity, design, feasibility *etc*. (Cox et al., 2017).

3 CRISPR/Cas systems

Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR), refers to the short repeating DNA sequences in the genome of prokaryotes. These sequences were first identified in *E. coli*, by Dr. Ishino's group in 1987, and since then these have been discovered in different prokaryotes (Mojica, Diez-Villasenor, Soria, & Juez, 2000). When a prokaryote is infected by a virus the repeated sequences are transcribed into CRISPR RNAs (crRNAs) that guide the CRISPR associated (Cas) proteins to break the plasmid or viral RNA/DNA sequences. Therefore, the CRISPR/Cas system is referred to as the adaptive immune system of prokaryotes (Jinek et al., 2012). The CRISPR/Cas system is classified into class1 and class2, and has six types (I-VI) with a continuously expanding list of at least 33 subtypes (Makarova, Wolf, & Koonin, 2018). The hijacking of CRISPR/Cas9 system (class2; type II) as molecular scissors for genome editing led the royal Swedish academy of sciences to award the Nobel prize in chemistry (2020) to Emmanuelle Charpentier and Jenifer A. Doudna (https://www.nobelprize.org/prizes/chemistry/2020/press-release/). Mechanistically, the CRISPR/Cas systems primarily require two parts; a nucleic acid binding domain, that recognizes the specific sequence and an effector protein (Cas) that cleaves/regulates nucleic acids. The basic mechanistic steps in all the CRISPR/Cas systems described till date are almost the same but the Cas proteins involved in the processing exhibit substantial diversity. Since its discovery, this system has been harnessed for a wide variety of applications, including the development of novel diagnostic and therapeutic approaches against infectious diseases. In this chapter, we present the use of different CRISPR/Cas systems for the potential development of advanced diagnostic and therapeutic strategies for SARS-CoV-2 4 CRISPR-based diagnostics for SARS-Cov-2.

The CRISPR/Cas systems have an immense capability of transforming the status of diagnostics and health care systems (Gootenberg et al., 2017) which are presently ready to take advantage of this technology. The principles of "collateral cleavage activity" have been exploited by recently developed CRISPR-based diagnostics in which the developers have made fluorescently labelled ssDNA/RNA reporter probes to detect the visible bands through the lateral flow assay in a paper strip, for the development of a novel nucleic acid-based diagnostic tool

(Chen et al., 2018; Zhang, Abudayyeh, Gootenberg, Sciences, & Mathers, 2020). The Cas effectors like Cas12a or Cas13 nuclease possessing collateral activity (described in detail in the next section on therapeutics) are becoming the most popular. Amongst these the Cas12 effectors are more effective in the detection of tumour associated viral markers, such as HPV (Chen et al., 2018) and the Cas13 effectors are better at RNA detection for viruses like Zika and Dengue (Gootenberg et al., 2017). Using this technology, different Cas proteins are exploited for the development of very efficient diagnostic kits for the diagnosis of COVID-19. The various types of Cas systems being explored for COVID-19 diagnosis are discussed in the next sections of this chapter.

3.1 Cas12

The different approaches developed using various types and sub-types of the Cas12 system are discussed in this section.

3.1.1 Cas12a

Different methods developed using the Cas12a system are discussed in the following sub-sections and summarized in Table 1.

Table 1 A summary of different approaches/methods developed using Cas12a system.

Method	Target gene in SARS-CoV-2	Remarks
DETECTR (Broughton et al., 2020)	Envelop (E) and Nucleoprotein (N)	Uses RT-LAMP for reverse transcription and isothermal amplification. Given Emergency Use Authorization (EUA) by FDA.
AIOD-CRISPR (Ding et al., 2020; Zhang et al., 2020)	Nucleoprotein (N)	Separate nucleic acid preamplification and multiple manual operations are required. One-pot reaction system with visual detection.
NER (Wang et al., 2020)	Orf1a, Orf1b, Nucleoprotein (N) and Envelop (E)	SARS-CoV-2 nucleic acid detected as green fluorescence under 485 nm light. Portable with high sensitivity and specificity.
iSCAN (Ali et al., 2020)	Envelop (E) and Nucleoprotein (N)	RT-LAMP coupled with CRISPRCas12a. Suitable for large-scale and early detection of SARS-CoV-2 carriers.
VaNGuard (Ooi et al., 2021)	N-gene	Highly efficient for detecting viral mutations. Robust, time and cost efficient, sensitive, specific, convenient point-of-care test for SARS-CoV-2.

3.1.1.1 DNA endonuclease-targeted CRISPR trans reporter (DETECTR)

The DETECTR system has been successfully used to differentiate between human papillomavirus 18 (HPV18) and human papillomavirus 16 (HPV16) from clinical samples as well as in the crude DNA isolated from cultured human cells within an hour (Myhrvold et al., 2018). It simultaneously performs the reverse transcription and isothermal amplification using the loop mediated amplification (RT-LAMP) and specifically recognizes the viral sequence *via* Cas12, leading to the cleavage of fluorescent and lateral flow reporter ssDNAs (Ramachandran et al., 2020). The main steps of this procedure include, (a) RNA extraction from nasopharyngeal/oropharyngeal swabs in the universal transport media (UTM), (b) Cas12 detection of pre-established coronavirus sequences, and (c) Cleavage of the reporter molecule (confirming virus) (Broughton et al., 2020). It has been demonstrated on predicted SARS-CoV-2 sequences initially and further studied on many clinical samples. This assay takes less than 40 min to complete and its limit of detection (LoD) is noted to be 10 copies/μL (Broughton et al., 2020). The primers for this test were designed to amplify the E (envelope) gene, having overlaps with the WHO assay (E gene region) and N (nucleoprotein) with US CDC assay (N2 region in the N gene) of SARS-CoV-2. The N1 and N3 regions lack the suitable PAM sites for the Cas12 gRNAs, therefore these were not targeted for amplification (Safari et al., 2021). This test was given EUA (emergency use authorization) by the FDA recently, provided it must only be used at a single centre (https://www.fda.gov/media/139934/download). Further studies on multi-centre comparison using clinical samples (Brandsma et al., 2020) suggested that the DETECTR method was demonstrated to be an efficient and rapid point-of-care (POC) test, with comparable sensitivity and specificity to RT-qPCR. Some limitations of this technique include the carry over contamination, need of nucleic acid extraction, kits and reagents, and requirement of personal protective equipment (Rahimi et al., 2021).

3.1.1.2 All-in-one dual CRISPR-Cas12a (AIOD-CRISPR) assay

The CRISPR-Cas-based nucleic acid detection method usually needs individual nucleic acid pre-amplification and multiple manual operations, that potentially increases the risk of carry-over contaminations due to amplified products shifting. In the AIOD-CRISPR assay, all the ingredients required for the nucleic acid amplification and CRISPR dependent detection are meticulously mixed in a one-pot reaction system, and incubated at a fixed temperature, to eliminate the requirement for individual pre-amplification and transfer of amplified product (Ding, Yin, Li, Lalla, et al., 2020). The target sequence for SARS-CoV-2 in this method includes a 121 bp N gene fragment (GenBank accession MT688716.1) and the initiation of dual CRISPR-based nucleic acid detection with high efficiency done by dual crRNAs without protospacer adjacent motif sites (PAM). The primers and crRNA of the AIOD-CRISPR assay were designed by selecting four sites of the target sequence, which were found to be highly conserved using the GISAID's multiple sequence alignment analysis ($n = 4663$) of SARS-CoV-2 genomes (https://www.gisaid.org/epifluapplications/next-hcov-19-app). The projected LOD of AIOD-CRISPR is about 4.6 copies for RNA targets and 1.2 copies for DNA targets in a

40 min incubation. This assay has been implemented to identify the genomic RNA of HIV and SARS-CoV-2 with high sensitivity within an hour (Ding, Yin, Li, Lalla, et al., 2020; Zhang et al., 2020). It was corroborated by testing 28 COVID suspected clinical swab samples where its ultra- specificity is demonstrated by detection of HIV-1 with negligible background as compared to the reported real-time RPA (Safari et al., 2021). The results were found to be consistent with that of the RT-qPCR method and is developed for rapid, simple, specific, ultrasensitive, one-pot, and visual detection of SARS-CoV-2. In addition, it replaced the need for a big incubator with a low-cost hand warmer leading to an instrument-free point of diagnostic method of COVID-19 (Ding, Yin, Li, Lalla, et al., 2020). A few limitations of the AIOD-CRISPR assay are the need for nucleic acid extraction, and the need for kits and reagents (Rahimi et al., 2021).

3.1.1.3 CRISPR/Cas12a-NER (naked eye readout)

Advances in diagnostic techniques has led to a more rapid and accurate method to detect SARS-CoV-2 nucleic acid using the CRISPR/Cas12a-NER system, the Cas12a protein, SARS-CoV-2 specific CRISPR RNAs (crRNAs) and a single-stranded DNA (ssDNA) reporter labelled with a quenched green fluorescent molecule. This reporter molecule is cleaved by Cas12a when there is SARS-CoV-2 nucleic acid in the detection system leading to green fluorescence clearly seen with the naked eye under 485 nm light (Wang et al., 2020). There are 15 crRNAs designed on four domains of the orf1a, orf1b, N and E genes over the Wuhan-Hu-1 strain (GenBank accession number MN908947) that can distinguish other SARS-related viruses on the basis of single nucleotide polymorphisms (SNPs). Results revealed that 14 crRNAs, except the *E*-crRNA1, targeting SARS-CoV-2 were validated, and were highly specific. Clinical validation of CRISPR/Cas12a-NER showed that it has 100% agreement with the results of RT-qPCR assays confirming the high performance of this platform (Wang et al., 2020). The portability, simplicity, sensitivity, specificity, no need for special instruments, time-efficiency, and visibility of results with the naked eye are some of the significant advantages of this method. However, the need for nucleic acid extraction with availability of automated extraction equipment, kits and reagents remain unaddressed drawbacks (Rahimi et al., 2021).

3.1.1.4 iSCAN (*in vitro* specific CRISPR-based assay for nucleic acids detection)

The iSCAN system involves the RT-LAMP coupled with CRISPR/Cas12 for the rapid, specific, accurate, sensitive detection of SARS-CoV2. Its development targeted two regions in N (at the highly conserved 3'end) and E genes wherein the identified primer set efficiently amplify the synthetic virus fragments, but not the controls. The LAMP primers were generated to ensure a robust amplification to suffice the LAMP-based detection *i.e.,* ~200 bp amplification products. This approach is specifically suitable for large-scale and early detection of SARS-CoV-2 carriers, allowing the effective isolation of individuals to limit the spread of the virus.

Ali et al. validated the detection kit using extracted RNAs from clinical samples of COVID-19 positive patients (Ali et al., 2020). A requirement of only rapid, field deployable, simple equipment is highly advantageous in using iSCAN, as the specific and easy to use RTLAMP and CRISPR/Cas12 takes less than 1 h (colorimetric reaction coupled to lateral flow immunochromatography makes easy interpretation of the results). However, the need of kits and reagents remains unsolved (Rahimi et al., 2021).

3.1.1.5 Variant nucleotide guard (VaNGuard) assay

This assay is highly efficient for viral mutations and can be utilized on purified RNA or directly on nasopharyngeal (NP) swab samples (Ooi et al., 2021). It includes three steps, *viz*. sample preparation, RT-LAMP reaction, and Cas12a based detection *via* fluorescence or lateral flow assay. The sample preparation requires proteinase K digestion of NP swabs and heat inactivation. The purified RNA or digested NP swab samples are mixed as the templates into RT-LAMP reactions which is followed by an incubation at 65 °C for 22 min. The enAsCas12a and ssDNA-probes are added after the incubation to lead to a further incubation at 60 °C for another 5 min.

The end-point fluorescence in this assay may be spotted by a plate reader or a RT-qPCR machine. Otherwise, a lateral flow strip may be implanted into each reaction tube for an equipment-free read-out. (Ooi et al., 2021). This assay is a robust, rapid, sensitive, affordable, specific, convenient point-of-care test for SARS-CoV-2. However, the need for nucleic acid extraction, kits and reagents are among the disadvantages (Rahimi et al., 2021).

3.1.2 Cas12b

Different methods developed using the Cas12b system are discussed in the following sub-sections and summarized in Table 2.

Table 2 A summary of different approaches/methods developed using Cas12b system.

Method	Target gene in SARS-CoV-2	Remarks
STOP (Joung et al., 2020)	N-gene	Appropriate for point-of-care applications. Detection of SARS-CoV-2 in about 1 h. High sensitivity, cost efficiency, convenient components, no need for RNA extraction.
CASdetect (Guo et al., 2020)	RdRp	Cas12b-mediated DNA detection range of detection was 1×10^4 copies/mL with reduced false positive rate. No cross-reactivity to other human endemic coronaviruses.

3.1.2.1 STOP (SHERLOCK testing in one pot)

Recently, this assay was developed as a simple test for detection of SARS-CoV-2 in about 1 h, that is appropriate for point-of-care applications. As compared to RT-qPCR-based SARS-CoV-2 tests, the sensitivity of STOP COVID has the LOD of 100 copies of viral genome/μL. The test results are obtained in 70 min with a dipstick, and in 40 min with a fluorescence readout. To make the test less complex Zhang et al. developed a simple protocol that does not require the sample extraction as it lyses the viral particles with QuickExtract at room temperature (22 °C) or in one-pot at an incubation temperature of 60 °C for 10 min (Zhang et al., 2020). The Cas12b is from *Alicyclobacillus acidiphilus* (AapCas12b) that sustains sufficient activity at the same temperature range as LAMP (55–65 °C). It is utilized for the detection of the N gene of SARS-CoV-2 in this defined assay. Since the AapCas12 locus lacks a CRISPR array the AapCas12b was integrated with the scaffold of *Alicyclobacillus acidoterrestris* Cas12b (AacCas12b) as tracrRNA (Trans-activating CRISPR RNA). The validation test on the clinical samples showed this assay successfully diagnosed 12 positive and 5 negative COVID-19 patients, with a minimum 2 of 3 replicates scoring positive in infected persons (Joung et al., 2020). The application of this platform may considerably help "test-trace-isolate" approach, particularly in the low-resource areas (Joung et al., 2020). Its simplicity, suitability for point-of-care (POC) analysis, sensitivity, cost efficiency, handiness of its components, no need of RNA extraction is among the main advantages of this assay whereas its disadvantages are negligible (Rahimi et al., 2021).

3.1.2.2 CASdetect (CRISPR-assisted detection)

Another diagnostic assay based on CRISPR, called Cas12b-mediated DNA detection (CDetection), was developed for the detection of SARS-CoV-2 (Guo et al., 2020). The detection range of the CASdetect system for SARS-CoV-2 pseudovirus was 1×10^4 copies/mL, without cross-reactivity to other human endemic coronaviruses. Incorporation of the sample treatment protocols and the nucleic acid amplification strategies with CDetection, an integrated viral nucleic acid detection system CASdetect (CRISPR-assisted detection) was developed (Guo et al., 2020). Some of the advantages of this tool are no cross-reactivity, reduced false positive rate, and accuracy whereas disadvantages are the need for nucleic acid extraction, need of kits and reagents (Rahimi et al., 2021).

3.1.3 Cas13

Different methods developed using Cas13 system are discussed in the following sub-sections and summarized in Table 3.

3.1.3.1 Specific high-sensitivity enzymatic reporter unlocking (SHERLOCK)

The SHERLOCK (Gootenberg et al., 2017) approach exploited Cas13a for the detection of RNA molecules. Later, a diagnostic platform based on this method was developed in 2018 (Gootenberg et al., 2018). This tool was employed for the detection of COVID- 19 (Zhang et al., 2020), to develop an improved and specific

Table 3 A summary of different approaches/method developed using Cas13 system.

Method	Target gene in SARS-CoV-2	Remarks
SHERLOCK (Gootenberg et al., 2017)	S-gene and Orf1ab gene	Specific High-Sensitivity Enzymatic Reporter Unlocking. Exploited Cas13a for the detection of RNA molecules. Amenability to automation, the use of a minimum volume of reagents, no need for sophisticated equipment.
CREST (Rauch et al., 2021)	N-gene	Cas13-based, Rugged, Equitable, Scalable Testing. Cas13 detection is integrated with thermal cycling amplification. Potential to pick up positive cases at early stages.
SHINE (Myhrvold et al., 2018)	ORF1a	SHERLOCK and HUDSON Integration to Navigate Epidemics. Need to prepare multiple reaction mixtures and handling multiple samples. Single-step reaction, no need for hospitals/ laboratories.
CARVER (Freije et al., 2019)	Putative genes N, E, RdRp or ORF	Cas13-assisted restriction of viral expression and readout. An end-to-end platform that uses Cas13 to detect and destroy viral RNA. Immense potential for diverse utility of rapid diagnostic and antiviral drug development.

diagnostic kit for COVID-19 to two targets - one from the S-gene and the other from the Orf1ab gene. The primers and gRNAs (LwaCas13a CRISPR) were developed to detect COVID-19 RNA (not cross reacting with related viral genomes) (Hou et al., 2020). This approach implements a non-targeted reporter RNA tagged to a fluorescent dye for the identification of specific RNA molecules (Kellner, Koob, Gootenberg, Abudayyeh, & Zhang, 2019). A web resource containing CRISPR-Cas13 based assay designs has been developed to identify 67 viruses, including SARS-CoV-2, Zika virus, and dengue virus, capable of selecting single or multiplex panels (Chen et al., 2018). The sensitivity of COVID-19 target sequences using SHERLOCK method is estimated in a range between 10 and 100 copies per microliter of input (20 and 200 aM), *i.e.*, LoD was 10–100 viral RNA copies/μL.

The SHERLOCK COVID-19 detection protocol can be completed in 1 h involving the following steps:

(1) An isothermal amplification of the sample (25 min incubation) with the help of recombinase polymerase amplification (RPA) kit.

(2) Identification of pre-amplified viral RNA with Cas13 (30 min incubation)
(3) Read out of the outcome with paper dipstick (2 min incubation)

SHERLOCK was validated on 154 clinical samples, with 96% and 88% sensitivity for the fluorescence and lateral flow readouts, respectively (Patchsung et al., 2020). Additionally, both the assays had 100% specificity (Patchsung et al., 2020). The advantages of SHERLOCK include amenability to automation and the use of a minimum volume of reagents, rapid, sensitive, and no need for sophisticated equipment (Rahimi et al., 2021).

3.1.3.2 CREST (Cas13-based, rugged, equitable, scalable testing)

To minimize the blockade to COVID-19 diagnostics, a method called CREST (Cas13-based, Rugged, Equitable, Scalable Testing) was devised (Rauch et al., 2021). The CREST platform with Cas13 detection is integrated with a thermal cycling amplification step (PCR), a linear amplification step (transcription), and enzymatic signal amplification *via* fluorescence detection. It eliminates the 3 main barriers, *viz*. reagent accessibility, equipment availability, and cost. Therefore, this tool has been developed by harnessing the advantage of widely available enzymes, low-cost thermocyclers (more cost effective than RT-qPCR), and easy-to-use fluorescent visualizers. Moreover, the CREST is equivalent in sensitivity (LOD-10 copies of a target RNA molecule per microlitre) to the reverse transcription quantitative polymerase chain reaction (RT-qPCR) method for COVID-19 testing. It therefore has the potential to pick up positive cases earlier than regular tools (Rauch et al., 2021). The advantages of CREST include that it can be executed from RNA sample to result without any requirement of a dedicated facility, within ~2 h, relieve some of the strain on the global supply chain for testing reagents, does not need specialized instrumentation, and requires very little specialized training. A few disadvantages are that, it requires nucleic acid extraction, needs kits and reagents (Rahimi et al., 2021).

3.1.3.3 SHINE (SHERLOCK and HUDSON integration to navigate epidemics)

To eliminate the need of nucleic acid extraction by using heat and chemical reduction, SHERLOCK can be combined with HUDSON (Heating Unextracted Diagnostic Samples to Obliterate Nucleases), for both viral particle lysis and elimination of RNA-degradation (Myhrvold et al., 2018). The combined SHERLOCK and HUDSON can be carried out with minimal infrastructure because only a heating element is required. But, the need to prepare multiple reaction mixtures and handling multiple samples in between are limitations. To address the current limitations of nucleic acid diagnostics, a special tool has been developed, named SHINE (SHERLOCK and HUDSON Integration to Navigate Epidemics) for extraction-free, rapid, and sensitive detection of SARSCoV-2 RNA. It has been shown that SHINE can identify SARS-CoV-2 RNA in HUDSON-treated samples (clinical) with either a paper-based colorimetric readout, or an in-tube fluorescent readout that can be carried out with portable equipment and reduced risk of sample contamination. Its advantages

include sensitivity, specificity, single-step reaction, no need for hospitals, laboratories and nucleic acid extraction, whereas its disadvantages are negligible (Rahimi et al., 2021).

3.1.3.4 CARVER (Cas13-assisted restriction of viral expression and readout)

CARVER is an end-to-end platform that uses Cas13 to detect and destroy viral RNA. Hundreds of crRNAs along with the LCMV (lymphocytic choriomeningitis virus) genome were screened to assess how conservation and target RNA nucleotide content influences Cas13's anti-viral activity (Freije et al., 2019). CARVER was also used to detect RNA viruses such as, influenza A and vesicular stomatitis, providing examples of its potential expanded application for the detection of a broad range of viral nucleotides in disease diagnosis (Freije et al., 2019). Hence, it is quite clear that Cas13 can be utilized to target a wide range of ssRNA viruses and CARVER has immense potential for diverse utility of rapid diagnostic and antiviral drug development.

3.1.4 Cas3

3.1.4.1 Cas3-operated nucleic acid detection (CONAN)

A combination of RT-LAMP and Cas3-based nucleic acid detection, resulted in an approach known as (CONAN) for COVID-19 diagnosis. Using post RNA isolation from clinical samples, RT-LAMP was carried out for SARS-CoV-2, and the leading amplicons were targeted using Cascade/Cas3 to achieve a fluorescence or lateral flow readout. The lateral flow-based CONAN approach was implemented on 31 clinical samples and showed 90% sensitivity and 95% specificity compared to the RT-qPCR assay. Similar results were reported with the DETECTR method (Broughton et al., 2020). Although, Cas3 was implemented for pathogen recognition for the first time, efficiency was at a level comparable to that of the Cas12a-based detection method, which has been in use for more than 2 years (Chen et al., 2018). Therefore, an even more sensitive SARS-CoV-2 detection method can be created by further optimization of the CONAN method. Advantages of this assay are time and cost efficiency, highly sensitive, and efficient single-base-pair discrimination. Whereas, disadvantages are nucleic acid extraction, and need of kits and reagents (Rahimi et al., 2021).

3.1.5 Cas9

3.1.5.1 FnCas9 editor linked uniform detection assay (FELUDA)

This class of CRISPR/Cas approaches for nucleotide recognition is dependent on the specific binding and cutting activity of CRISPR/Cas9. Previously, this method was used for the detection of Zika virus. Explicitly, Cas9 from *Francisella novicida* (FnCas9) was found to be highly specific for both target DNA binding and cleavage (Acharya et al., 2019). Its high specificity helped in developing a FnCas9-based nucleic acid detection method named (FELUDA) (Azhar et al., 2020), which was quickly adapted by these authors for diagnosis of COVID-19. This method used synthetic DNA fragments coding N gene of SARS-CoV-2, demonstrating that this

Table 4 List of diagnostic methods that can be used for diagnosis of COVID-19.

S. No.	Method of diagnosis	Cas enzyme	LOD[a] (limit of detection)	References
1	DETECTR	Cas12a	10 copies/μL	Broughton et al. (2020)
2	AIOD-CRISPR	Cas12a	RNA −4.6 copies/μL DNA −1.2 copies/μL in 40 min	Zhang et al. (2020) and Ding, Yin, Li, Lalla, et al. (2020)
3	CRISPR/Cas12a-NER	Cas12a	10 copies/μL	Wang et al. (2020)
4	iSCAN	Cas12a	10 copies/μL	Ali et al. (2020)
5	VaNGuard	Cas12a	20 copies/μL	Ooi et al. (2021)
6	STOP	Cas12b	100 copies	Joung et al. (2020)
7	CASdetec	Cas12b	1×10^4 copies/mL	Guo et al. (2020)
8	SHERLOCK	Cas13	10–100 viral RNA copies/μL	Gootenberg et al. (2018)
9	CREST	Cas13	10 copies/μL	Rauch et al. (2021)
10	SHINE	Cas13	10 copies/μL	Myhrvold et al. (2018)
11	CARVER	Cas13	Yet to comprehend	Freije et al. (2019)
12	CONAN	Cas3	1 copies/μL	Chen et al. (2018)
13	FELUDA	Cas9	∼10 copies/μL	Azhar et al. (2020)

[a]*Each molecular method developed to detect COVID-19 is mentioned with its respective limit of detection (LOD) and the Cas enzyme used.*

assay can identify SARS-CoV-2 and is capable of distinguishing it from SARS-CoV and H1N1 viral sequences. This method was validated by screening total RNA of COVID-19 patient samples within 45 min. It is also claimed that FELUDA was compatible with fluorescence readout and with RT-RPA as a pre-amplification method (Azhar et al., 2020). Thereafter, the FELUDA method was adapted for the lateral flow readout by using the catalytically inactive form of FnCas9 (dFnCas9), FAM-labelled trans-activating CRISPR RNA (tracrRNA), and biotinylated PCR primers (Azhar et al., 2020). A summary of the limits of detection (LOD) and the Cas enzyme used for all the diagnostic methods discussed above is provided in Table 4.

4 CRISPR-based therapeutics for SARS-Cov-2

The replication of viral nucleic acid inside the host cells is one of the key steps required for completion of successful viral life cycle. Targeting viral genes and rendering the virus non-replicative is considered as an ideal therapeutic strategy.

For decades, the biggest hurdle for targeting the viral genome was the lack of precise gene editing techniques. With the advent of the CRISPR/Cas systems, manipulation of virus genes has been tested for therapeutic studies in many viruses (Freije et al., 2019; Lee, 2019). Likewise, CRISPR/Cas systems can be utilized in different ways to develop anti-SARS-CoV-2 strategies.

4.1 Disrupting the viral RNA genome

Most of the antiviral approaches target the proteinaceous structural or non-structural components and inhibit the virus at one of the viral life cycle stages (attachment, entry, uncoating, replication, assembly, release). Around the world, CRISPR/Cas systems have been adopted by the scientific community to limit viral replication by targeting the viral genome. The CRISPR/Cas9 has proven its potential as an anti-viral strategy for many DNA viruses, *in-vitro* as well as *in-vivo* (reviewed by Lee, 2019). Likewise, the CRISPR/Cas12a system (from *Lachnospiraceae bacterium*) is demonstrated as a promising tool to inactivate integrated HIV DNA genomes in cell culture. Gao and co-workers also reported that Cas12a outperformed Cas9 for HIV inhibition (Gao, Fan, Das, Herrera-Carrillo, & Berkhout, 2020). Moreover, unlike Cas 9 and Cas12a CRISPR enzymes, which have the capability to manipulate DNA, the CRISPR/Cas 13 system has been identified as a novel RNA guided RNA targeting system (Abudayyeh et al., 2017). The Cas13 system can recognize and degrade the viral genome of SARS-CoV-2 by targeting positive sense single stranded genomic RNA as well as viral mRNA formed by genomic and subgenomic RNA, to limit virus replication. Identification of the RNA targeting activity of the CRISPR/Cas13 system has immensely increased its potential applications in terms of research and development of new anti-viral therapies and has the capability for development of anti-SARS-CoV-2 therapies.

4.1.1 Overview of Crispr/Cas13 system

The CRISPR/Cas13 belongs to Class2; type VI CRISPR Cas family (the only known CRISPR nuclease with a single-effector, exclusively targeting single stranded RNA) with a minimum of four subtypes recognized; VI-A, Cas13a (Class2candidate2/C2c2); VI-B, Cas13b (C2c6); VI-C, Cas13c (C2c7); and VI-D, Cas13d (Abudayyeh et al., 2017; Cox et al., 2017; Lin et al., 2021). The Cas13a was used as an RNA targeting enzyme (Abudayyeh et al., 2016) whereas Cas13b has been adapted as a precise RNA editing enzyme (Cox et al., 2017). Like Cas9, Cas13a consists of one crRNA recognition (REC) lobe and a nuclease (NUC) lobe which contains various functional domains (O'Connell, 2019). The enzymes in the Cas13 family show two different catalytic activities: (a) RNA cleavage, mediated by two RNase motifs (R-X_4-H) which are conserved among all four subtypes that belong to the superfamily of higher eukaryotes and prokaryotes nucleotide (HEPN) binding domains (Zhang et al., 2018), (b) a guide RNA (gRNA) maturation activity (pre-crRNA processing), probably due to activities in the Helical-1 and HEPN2 domains (Abudayyeh et al., 2017).

4.1.2 Mechanism of action of CRISPR/Cas13 system

Mechanistically, the Cas13 enzymes require a 60–66 nucleotide long crRNA which recognizes a 28–30 nucleotide long sequence on the target RNA. The crRNA is a short hairpin structure and forms a complex with Cas13 enzyme like Cas9 enzyme. Unlike Cas9, Cas13 does not require a protospacer adjacent motif (PAM) sequence at the target locus. This property makes Cas13 system more flexible to use. Some members of the Cas13 system require a protospacer flanking sequence (PFS) located near the protospacer (not named PAM because it is used for a sequence used in self and non-self-differentiation whereas PFS is not) which varies with the subtype (Marraffini & Sontheimer, 2010). For example, subtype VI-D and a few members of VI-A do not require PFS (Burmistrz, Krakowski, & Krawczyk-Balska, 2020). Unlike Cas9 endonuclease that cleaves the dsDNA and reverts back to the inactive stage once DNA is cleaved, Cas13-crRNA complex gets activated by binding to its target RNA and cleaves the target ssRNA as well as the other RNAs non-specifically, resulting in "collateral cleavage" activity (Abudayyeh et al., 2016). The property of collateral damage is independent of the presence of PFS or homology to the crRNA and was utilized for diagnostic purposes. For example, SHERLOCK (Myhrvold et al., 2018) *etc*. that has been described above in the diagnostic section. This bystander RNase activity is a general property of the class2 system which targets ssRNA. Some different subtypes of Cas13 have been studied for their antiviral potential. A few of these studies are described under the heading, applications in next section (Section 4.1.3).

4.1.3 Applications of Cas13 variants as potential antivirals

4.1.3.1 Cas13a

Cas13a was originally identified in 2015 by Shmakov and co-workers, as *Leptotrichia shahii* (LshCas13a) which utilizes a short 24 nt long crRNA. Unlike the most widely used CRSIPR/Cas9 system which needs crRNA and trcrRNA (as a single guide RNA (sgRNA)), Cas13a utilizes only a crRNA. The crRNA interacts with the LshCas13a *via* a Uracil rich stem loop and requires a PFS consisting of A, C or U base pair, located at the 3′ end of the spacer sequence (Shmakov et al., 2015). The Zhang lab at the Broad Institute of MIT and Harvard discovered that some orthologues of Cas13 can be used in mammalian and plant cells (Abudayyeh et al., 2017). Cas13a from *Leptotrichia wadei* (LwaCas13a) is well characterized and it does not require a PFS at the target site, making it more flexible for therapeutic applications. However, it requires stabilization by a monomeric superfolder green fluorescent protein (GFP) for effective RNA knockdown in mammalian cells (Cox et al., 2017). Moreover, the LwaCas13a does not display collateral cleavage activity in eukaryotic cells. For Cas13a, the processing of pre-crRNA into mature crRNA is not required, as pre-crRNAs themselves can acts as a guide for the cleavage of the ssRNA target sequence (East-Seletsky, O'Connell, Burstein, Knott, & Doudna, 2017).

Blanchard and colleagues demonstrated that the specific activity of Cas13a to knock down endogenous genes can be utilized for treatment of respiratory viral infections including SARS-CoV-2 infections (Blanchard et al., 2021). They performed

a prophylactic as well as a therapeutic study in VeroE6 cells by targeting the conserved regions (Nucleocapsid and replicase) of SARS-CoV-2, using synthetic mRNA to express LbuCas13a (from *Leptotrichia buccalis*) and demonstrated that SARS-CoV-2 was inhibited. They also demonstrated an *in-vivo* mitigation of the virus in a Syrian hamster model by using an inhalation-based apparatus to deliver Cas13mRNA along with crRNA. In another study, with the help of bioinformatics tools, crRNAs against the SARS-CoV-2 receptor binding domain (RBD) were identified and their editing efficiency was evaluated in human epithelial type II (AT2) cells and human hepatocarcinoma cells (HepG2). They designed CRISPR/Cas13a (LwaCas13a) based technology against SARS-CoV-2 for targeting and cleaving its RNA and found that crRNA6 was a potential candidate (Wang et al., 2021). Apart from the cleavage of SARS-CoV-2 RNA (a strategy considered useful in early infection), they also used the same strategy for reducing viral proteins (more appropriate during late infections) using the dCas13a-crRNA6. DeadCas13a (dCas13a) is a catalytically inactive version of Cas13a, generated by causing R474A and R1046A mutations in the HEPN domains, which only binds with the target RNA sequence and does not cleave it, thus regulating the transcription of SARS-CoV-2 genes (Abudayyeh et al., 2017).

4.1.3.2 Cas13b

Like the Cas13a subtype, CRISPR loci encode a single effector protein, Cas13b which contains two predicted HEPN domains at its N and C- termini. The CRISPR/Cas13b system cleaves ssRNA using HEPN domains and also exhibits collateral RNase activity. Apart from HEPN domains there is no sequence similarity between 13a and 13b. Unlike Cas13a, Cas1 and Cas2 are absent in the Cas13b system and it can process its own CRISPR array. The Cas13b system has two variants denoted as VI-B1 and VI-B2 with their accessory proteins named as Csx27 and Csx28 respectively. In the VI-B1 system, the Csx27 causes repression of Cas13b whereas Csx28 enhances Cas13b activity. It has been shown that along with several factors, the presence of secondary structure in the RNA target impacts the activity of Cas13b enzymes (Smargon et al., 2017). A research group from Thailand showed that CRISPR/Cas13b system effectively abrogated the porcine reproductive and respiratory syndrome virus (PRRSV), by simultaneously targeting ORF5 and ORF7 genes (Cui, Techakriengkrai, Nedumpun, & Suradhat, 2020).

The antiviral potential of Cas13 systems (both 13a and 13b) have been evaluated in 3 different ssRNA viruses [lymphocytic choriomeningitis virus, (LCMV), Influenza A virus (IAV) and vesicular stomatitis virus (VSV)] in cell culture by Freije et al. (2019). They carried out a computational analysis followed by experimental validation of viral genome sequences to create a repository of antiviral crRNAs. They used Cas13a (LwaCas13a) and Cas13b (PspCas13b) from *L. wadei* and *Prevotella sp. P5–125*, respectively, to demonstrate the generalizability of the Cas13 system. Their study demonstrated that Cas13 can efficiently reduce the viral RNA levels in mammalian cells, confirming the potent antiviral activity of Cas13 against different ssRNA viruses (Freije et al., 2019). In comparison to the Cas13a system,

the CRISPR/Cas13b system was found to be more robust and its activity can be upregulated or downregulated depending on the accessory proteins encoded by its loci (Cox et al., 2017). Considering that SARS-CoV-2 also possess a ssRNA viral genome, the same principles can be applied to generate anti SARS-CoV-2 therapeutics.

4.1.3.3 Cas13d

For working with this system, guide RNA is made up of a 30 nt direct repeat with a 22 nt spacer (Fig. 1). Spacer length of 22 nt is desirable as below this length the cleavage activity is significantly dropped. In mammalian cells, the collateral cleavage activity of Cas13d is absent. Also, the target cleavage does not depend on the flanking sequence requirements but the cleavage pattern varies with the target. For example, *Eubacterium siraeum*/EsCas13d prefers uracil bases in the target region above all other bases. The study was conducted to find out an active Cas13d orthologue in eukaryotic cells and revealed that an engineered variant of Cas13d *Ruminococcus flavefaciens* strain XPD3002 (Rfx) Cas13d (CasRx) can be developed into a flexible tool for programmable ssRNA targeting in mammalian cells. They compared the small hairpin RNA (shRNA) interference, CRISPR subtype VI-A/VI-B and Cas9 mediated transcriptional inhibition (CRISPRi) with CasRx and found that CasRx outperformed with 96% knockdown as compared to 65% knockdown by shRNA, approximately 70% by CRISPRi and 53% by CRISPRi; suggesting CasRx was the most efficient RNA regulating method. Another study by Yan and co-workers, characterized *E. siraeum* (EsCas13d) and *Ruminococcus sp.* (RspCas13d) orthologues of Cas13d enzyme (Yan et al., 2018). They also showed that Cas13d associated accessory proteins have a WYL domain, because the target activity of RspCas13d was increased, indicating that this particular domain regulates the activity of Cas13d (Yan et al., 2018). Recently, a research group from Stanford University developed a potential CRISPR/Cas13d-based pan-coronavirus inhibition strategy. They named it prophylactic antiviral CRISPR in human cells (PAC-MAN) and showed that it could cleave the SARS-CoV-2 sequences that were effective as novel antiviral therapy against COVID-19 (Abbott et al., 2020). Abbott and colleagues used CasRx and synthesized 20 crRNAs targeting RNA-dependent RNA polymerase (RdRp) and Nucleocapsid (N) genes, each. They chose a Cas13d expressing human lung epithelial cell line (A549) to transduce the pool of crRNAs and found that the targeting RdRp and N gene repressed a reporter by 86% and 71% respectively. It was also demonstrated that the SARS-CoV-2 inhibition was quite sensitive to different crRNA concentrations and lowering the Cas13d expression has little effect on the inhibitory activity of the Cas13d system (Abbott et al., 2020). They also suggested that CRISPR/Cas13d can be used as an antiviral strategy, both prophylactically and therapeutically (Fig. 2).

Consolidating the studies on antiviral capabilities of subtypes of CRISPR/Cas13 systems, Cox and co-workers detected that among the different orthologues of Cas13a, b and c; Cas13b (PspCas13b) was the most specific and efficient for RNA knockdown in mammalian cells (Cox et al., 2017). LwaCas13a had two major

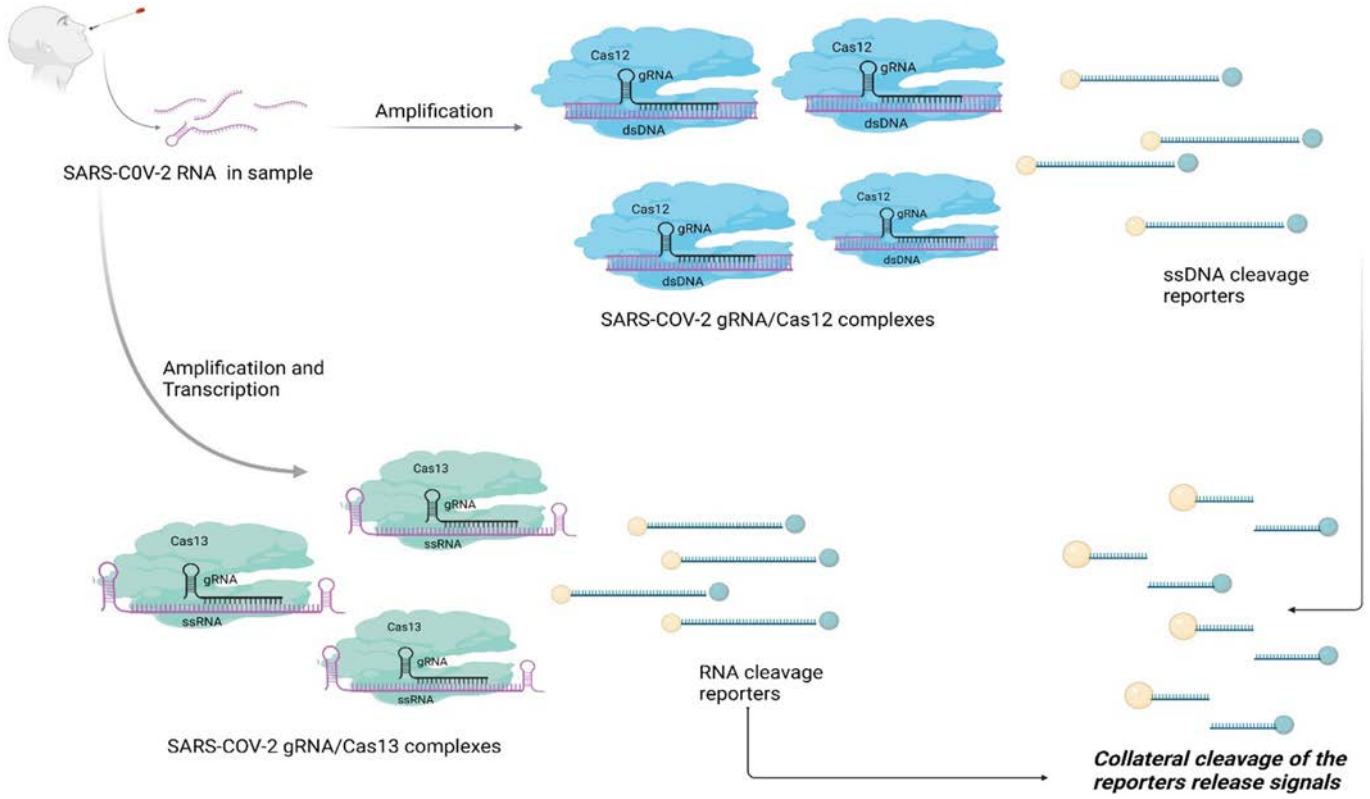

FIG. 1

Schematics for SARS-Cov-2 detection using CRISPR/Cas platform. The RNA is extracted from the patient using conventional RNA extraction method. For Cas12 based detection the RNA is amplified into dsDNA whereas for Cas13 based detection the amplified DNA is transcribed into ssRNA. The collateral activity of both Cas12 and Cas13 is used for the detection of SARS-CoV-2.

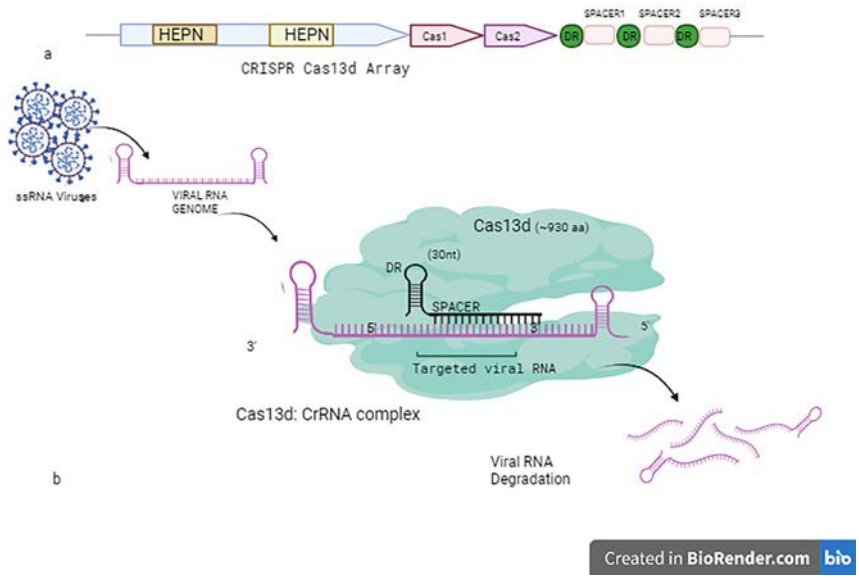

FIG. 2

(A) CRISPR/Cas13d array showing HEPN domains. (B) Schematic of CRISPR/Cas13d mechanism as an antiviral strategy. Cas13d shown as a green cloud and guide RNA (DR and spacer) together forms a Cas13d:crRNA complex required for viral RNA degradation. DR: Direct repeat.

issues; first it must be stabilized with monomeric super-folded GFP and secondly, the average RNA knockdown efficiency was around 50% whereas PspCas13b provided better efficiency as compared to LwaCas13a with 62.9% knockdown. Although these systems can be reprogrammed to target ssRNA, it is difficult to pack them into adeno-associated vectors (AAV) for *in-vivo* delivery due to their large size. In comparison to Cas13a (1250 aa), Cas13b (1150 aa), and Cas13c (1120 aa), Cas13d effector has an average length of 930 aa (the smallest class2 CRISPR effector) (Cox et al., 2017; Shmakov et al., 2015; Smargon et al., 2017). Overall, the CRISPR/Cas13d system seems to be the most practical system for a therapeutic approach to target the SARS-CoV-2 genome. The small but robust CRISPR/Cas13d system seems to be the latest technology for the RNA engineering toolbox.

This claim may be further supported by the following statements.

i. The size of Cas13d (approx. 930aa) is 17–26% smaller than other class2 type VI-CRISPR/Cas subtypes (Cas13a-c); which makes it suitable for packaging in low- capacity vectors, like AAV; making it particularly suitable for delivery in primary cells and *in-vivo* applications.
ii. It lacks any sequence constraints for flanking sequences *i.e.*, Cas13d does not require the presence of PFS. This property makes it possible to target theoretically any ssRNA sequence.

iii. Modular activity of WYL1 for CRSISPR/Cas13d system: CRISPR/Cas13d locus consists of an accessory protein with the WYL domain (named for three amino acids conserved in the original domain) (Yan et al., 2018). Co-occurrence of Cas13d with accessory proteins having a WYL-domain increases the targeted cleavage, implying such proteins act as regulators for Cas13d activity.
iv. Comparison between Cas13a/b effectors and Cas13d effector (CasRx) as RNA regulating systems, showed knockdown of approximately 70% by Cas13a/b enzymes whereas 96% by CasRx.

Therefore, the strong catalytic activity, high specificity, and small size of the Cas13d protein makes it the best choice for targeting the SARS-CoV-2 genome (Abbott et al., 2020). Studies showed that the highly conserved regions of SARS-CoV-2 [(Abbott et al., 2020) nucleocapsid (N) which protects the viral genome and (Abudayyeh et al., 2017) RNA-dependent RNA polymerase (RdRp) which catalyses the transcription of all viral mRNAs] can become potent targets of CRISPR/Cas13d (Chan et al., 2020), thus disabling virus production and function (Abbott et al., 2020).

4.2 Disrupting the host cell factors essential for SARS-CoV-2 infection

One of the key questions in virology has always been: how viruses that encode comparatively few genes, gain control over their host cells? The answer is partly because some host factors are utilized by the viruses at some stage(s) during their life cycle. Along with targeting SARS-CoV-2 genes, the identification of the host factors that promote or restrict the replication of novel viruses can lead to the recognition of new targets for antiviral therapeutics. For studying such interactions between virus and host, different techniques including forward genetic screens are being used. Although the CRISPR/Cas9 system is not the first technique to study genetic screens, it has certainly emerged as the most robust tool to date. Basically, in forward genetic screens, mutated genes are studied by changes in their phenotypes. Using genome scale CRISPR/Cas screens, the host genes which promote or limit the viral replication, can be identified within the entire host genome. Genome wide CRISPR screens are being carried out for SARS-CoV-2 by many researchers. For example, Wang and co-workers found that apart from the well-known angiotensin converting enzyme (ACE-2) entry receptors, other candidate host factors are TMEM106B, VAC14, cholesterol regulators, and subunits of exocysts, that support the infection of SARS-CoV-2 and thus can be targeted for development of potential antiviral strategies (Wang, Simoneau, et al., 2021). Likewise, CRISPR based genetic screens have found different candidate host genes (Daniloski et al., 2021; Wei et al., 2021; Zhu et al., 2021) which can be targeted by the CRISPR/Cas9 system to generate potential antiviral therapeutics for SARS-CoV-2.

5 Delivery of CRISPR/Cas components

Like the CRISPR/Cas9 system, the Cas13-crRNA also has different expression modalities which includes, ribonucleoproteins (RNPs), mRNAs and plasmids. Researchers are investigating different strategies to deliver the CRISPR components successfully into the target host. Owing to the small size of the Cas13d enzyme, viral delivery using adeno associated virus (AAV) can be considered as an *in-vivo* delivery platform, which has shown promise in the mouse model (van Lieshout, Domm, & Wootton, 2019). Also, a liposome- based gene delivery platform; high-level extended duration gene expression system (HEDGES) has been used to deliver the CRISPR components in immunocompetent mice. Unlike viral delivery, this system does not elicit any anti-vector immune response, host toxicity or DNA integration into the host genome (Handumrongkul et al., 2019). HEDGES can be used to deliver the crRNAs and the mRNA encoding the Cas13d. Krishnamurthy and co-workers demonstrated that the amphiphilic shuttle peptides can also be used to deliver the CRISPR components (as ribonucleoproteins/RNP) to cultured human epithelial cells and to mouse airway epithelia (Krishnamurthy et al., 2019). Likewise, RNP based delivery can also be performed using synthetic carriers like gold nanoparticles which have been tested in a mice model (Amirkhanov & Stepanov, 2019; Shahbazi et al., 2019). Guan and co-workers showed that the mRNA and plasmid DNA can be delivered using poloxamine-based copolymer (peptide poloxamine nanoparticle) *in-vitro* as well as *in-vivo* (mice lungs) genetic modifications (Guan et al., 2019). Despite all these developments, *in-vivo* delivery of the CRISPR components to a particular cell type of interest is still a matter of investigation. Delivery studies for the Cas13 system are in its infancy especially because Cas13 systems are only beginning to be discovered and characterized (East-Seletsky et al., 2016).

6 Limitations of CRISPR/Cas system

Although the Cas13d system holds the title of most promising tool for the development of anti-viral strategies against RNA viruses including SARS-CoV-2, there are a number of limitations which should be addressed before CRISPR/Cas13d technology can be introduced to medical clinics;

i. First and foremost is the lack of a safe and effective *in-vivo* delivery method into human respiratory tract cells: As the Cas13d enzyme has a small size, adeno-associated virus (AAV) seems to be the most feasible option but the adaptive immune response against AAV might be of a concern. Hopefully, one of the methods mentioned in Section 6 could be used for the CRISPR based antiviral delivery in humans using a nasal spray/nebulizer system, in the future.
ii. Off-target effects: An evaluation of the off-target activity of the crRNAs using whole transcriptome RNA sequencing would be required.

iii. Validation of the above-mentioned studies in pre-clinical animal models (macaques/ferrets) to test the specificity and efficacy of the CRISPR based antiviral strategies.
iv. The most advanced study among the above-mentioned studies is PAC-MAN but its major shortcoming is that it was done on synthetic constructs of the virus. With knowledge of the exact effects of the CRISPR/Cas13 system, validation could be done using live SARS-CoV-2.

7 Summary

Since the beginning of the COVID-19 pandemic, many diagnostic approaches (RT-qPCR, RAPID, LFA) have been adopted, among which RT-qPCR turned out to be the most popular/gold standard. But, one of the mystifying facets of COVID-19 is its presentation of a wide range of symptoms which varies among different patients and needs early diagnosis for better management. Even though RT-qPCR is a precise molecular technique false negative results may be obtained. On the other hand, CRISPR-based SARS-CoV-2 detection approaches are cost and time efficient, highly sensitive and specific, and do not require sophisticated instruments. Moreover, they also have shown promise to increase scalability and enable the diagnostic tests to be carried out at the point-of-care (POC). The CRISPR can be customized to the target for any genomic region of interest within the desired genome possessing a broad range of other applications and has been efficiently implemented for diagnosis of SARS-CoV-2. Considering the therapeutics, we need to understand that the traditional vaccines priming the immune system against the exposure to the viral proteins shows a high rate of mutations, overcoming the host immune response. In comparison, antiviral therapies targeting the highly conserved gene sequences can limit the escape of SARS-CoV-2 by the immune system. The CRISPR/Cas systems provide specific gene targeting with immense potential to develop new generation diagnostics and therapeutics. Moreover, with the CRISPR/Cas based therapeutics, multiplexing is possible, where different sgRNAs or crRNAs can be guided to more than one target within the same gene thus decreasing the possibility of viral escape mutants. As an exceptionally efficient tool the CRISPR/Cas13 system, CARVER (Cas13-assisted restriction of viral expression and readout) can be implemented to target a broad range of ssRNA viruses, and it can be used for both, diagnosis and treatment for a variety of viral diseases including SARS-CoV-2. However, the efficacy and safety of the CRISPR-based therapeutics needs to be assessed in pre-clinical and clinical settings. Although the CRISPR biotechnologies are not very helpful to control the present pandemic of COVID-19 there is hope that the limitations of the CRISPR/Cas system can be overcome in the near future. The CRISPR based strategies would lead to a new era in the field of disease diagnosis and therapeutic development, that would make us better prepared for future viral threats.

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