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CRISPR Cas system: A strategic approach in detection of nucleic acids

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ABSTRACT

Over the decades, rapid nucleic acid detection has been difficult for scientists, especially in microbiology, biotechnology and immunology. Most technologies are finite in sensitivity, specificity or both. Early diagnosis of disease allows swift response, disease monitoring and control of the rapid spread of the disease. However, limited access to test kits, specialized laboratory equipment, and the need for highly skilled personnel has led to a detection downshift. CRISPR-based diagnostic techniques, based on clustered regularly interspaced short palindromic repeats (CRISPR), have recently altered molecular diagnosis. This modern technology is combined or paired with other methods like SHERLOCK, DETECTR, HUDSON, CDetection and so on to detect viruses, bacteria infection and other pathogenic agents, due to its precision and versatility, it can also detect diseases directly from patient samples. Rapid, sensitive, accurate and advanced molecular techniques are much needed since they soothe researchers in diagnosis and detection, and can also be employed in therapeutic treatments. In this review, detailed CRISPR Cas variants were discussed, emphasizing the use of CRISPR system as a tool in diagnostics and nucleic acids detection and the benefits of this robust tool over other amplification methods.

1. Introduction

In several fields, such as microbial disease detection, quick, reliable, and accurate detection of specific nucleic acid sequences has become increasingly crucial (Lauri and AMariani, 2009). Recently, many detection technologies have been developed and widely applied in real-life applications including nanotechnology, interface modification technology, molecule assembly techniques and biomolecular recognition technology (Zhou et al., 2018). Throughout the last decade, researchers are exploring and have developed accurate and cost-effective molecular diagnostic technologies for detecting microbial infections. In recent researches, the authors stated the use of computer-aided detection (Artificial intelligent-driven models) to identify CRISPR-bio sensing techniques in pathogenic bacteria and viral disease. The study predicted the use of artificial intelligence as an affirmative approach that can help clinicians in terms of accuracy, cheap, and specificity in detecting microbial diseases (Umar Ibrahim et al., 2021). Also, in another study, the authors suggest the use of cloud computing and CRISPR-based biosensor as a futuristic approach for swift, and sensitive detection methods of pathogenic infections (Ibrahim et al., 2020).

Microorganisms are minute eukaryotic and prokaryotic pathogens that can affect most organisms on earth and are ubiquitous in nature (Greber and UFBartenschlager, 2017). Infections and diseases caused by these microbes are usually a threat to humans, leading to significant social and economic losses. Examples of some microbes that cause infection include viral infections like SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus type 2) (Zhang et al., 2020), bacterial infections like *Mycobacterium tuberculosis*, fungal infections such as skin diseases e.t.c. The control, monitoring, and management of infectious disease require the rapid, precise, and cost-effective detection and identification of pathogens (Niemz et al., 2011). On the other hand, standard advances, are required to build affordable point-of-care diagnostics that can reach people in underserved areas with limited access to healthcare. A diagnosis that necessitates the processing of materials in

Abbreviations: CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats.

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laboratories slows delay diagnosis, especially in locations where resources are scarce, infrastructure is lacking, and experienced medical personnel are scarce. However, for efficient disease treatment, creating reliable and quick diagnostic tests that can be employed outside of the clinical laboratory is critical for effective disease treatment and prognosis. Conventionally, nucleic acids and diseases can be detected by whole genome sequencing, DNA microarrays, hybridization-based detection methods e.t.c, and some disadvantages exist within these detection methods such as time-consumption, cost-effectiveness, cross-reactivity, skilled technical personnel dependence and expensive instruments (Pletz et al., 2011). Ideally, a precise approach for detection should be specific, sensitive, equipment-free and suitable for point of care testing (Ruiz-Vega et al., 2021). Nucleic acids were detected mainly by gene cloning strategies and hybridization procedures, PCR-based technique in amplification of nucleic acids has been the universal way of detecting pathogens in samples (Lazcka et al., 2007). PCR amplifies target genes exponentially and can detect single copies of pathogenic genomes. Despite the versatility of this technique, it still has some setbacks like lack of standardized protocols, lack of qualified personnel who rely on well-established laboratories and the treatment of reagents. Importantly, it cannot be used at point of care detection (Yang and SRothman, 2004). CRISPR systems, a contemporary and precise technique that has various applications can be employed for sensitive and specific detection of pathogen-specific nucleic acids, thereby unlocking the benefits for diverse in-field diagnostic and genotyping applications.

CRISPR Cas systems (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated) particularly, has intrinsic advantages of efficient nucleic acid recognition and editing abilities, have shown to be exceptional tools for specific nucleic acid detection (Li et al., 2019b), and allows easy application to detect nucleic acids associated with diseases. This technology, which is natively inhabitant of prokaryotes, provides adaptive immunity to bacteriophages and transformation of plasmid, advanced in modern technologies for diagnostics and treatments, played pivotal roles in therapeutic areas and has helped tremendously in, base editing, transcriptional regulation, gene editing, epigenome editing and especially in detecting nucleic acid that is vital to human health (Bhaya et al., 2011). CRISPR technology is a vast tool and not limited to nucleic acid detection; CRISPR Cas systems are dynamic and used compared to PCR and other amplification methods. They provide ultrasensitive identification of tests in infectious diseases, bacteria, tumor DNA, cancer-related diseases with easy processing. CRISPR Cas systems outperform PCR-based approaches not just because of the use of particular primers during isothermal amplification, but also because of the precision with which the Cas-sg/crRNA complex spots target templates. In this review, we discussed CRISPR Cas variants, the role of CRISPR Cas system in detecting of nucleic acids, provided some examples where CRISPR Cas proteins has been used in nucleic acids detection and stated the advantages of CRISPR systems over other amplification methods.

2. CRISPR Cas system

CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated) was discovered in 1987 and was described in the genome of bacteria (Ishino et al., 1987), known as the adaptive immune system of bacteria, used widely as a defensive mechanism in foreign nucleic acids, coupled with Cas proteins against invading foreign DNA and viruses in bacteria and archaea (Wiedenheft et al., 2012). When viruses infect bacteria, the bacteria produce a type of DNA that binds to virus DNAs. The Cas enzyme, which works with a nuclease called Cas, cuts the invaded DNA into pieces. Hence, the reason why it is an immune-defense mechanism in prokaryotes. (Fig. 1) illustrates the CRISPR Cas mechanism in a prokaryotic cell. This technology is one out of the recent advanced technologies that significantly impacted genetic engineering abilities and manipulations in past years. With this innovation, scientists decided to use this technology for gene editing using this naturally occurring immune defense system. Studies show that CRISPR Cas systems can be reconstructed in-vitro, while retaining their biological functions also have the ability to cut individual DNA sequence (Gasiunas et al., 2012). This paved the bedrock of CRISPR as a genome-editing tool. The study further shows that for CRISPR to be used as an editing tool, three components are required namely, Cas enzyme, crRNA and tracRNA. Single guide RNA (sgRNA) can be made by combining CRISPR RNA (crRNA) with trans-activating crRNA (tracRNA) to generate a single chimeric synthetic RNA molecule that works flawlessly for genome editing (Jinek et al., 2012). In comparison to prior gene editing tools ZFNs and TALENs, this research makes the CRISPR Cas system a straightforward technique to manipulate genes. Through the wide use of these CRISPR based tools, novel diseases tactics and pathogen/infection treatments have been designed and manipulated to save lives.

Primarily, CRISPR-Cas systems have their Cas proteins also known as

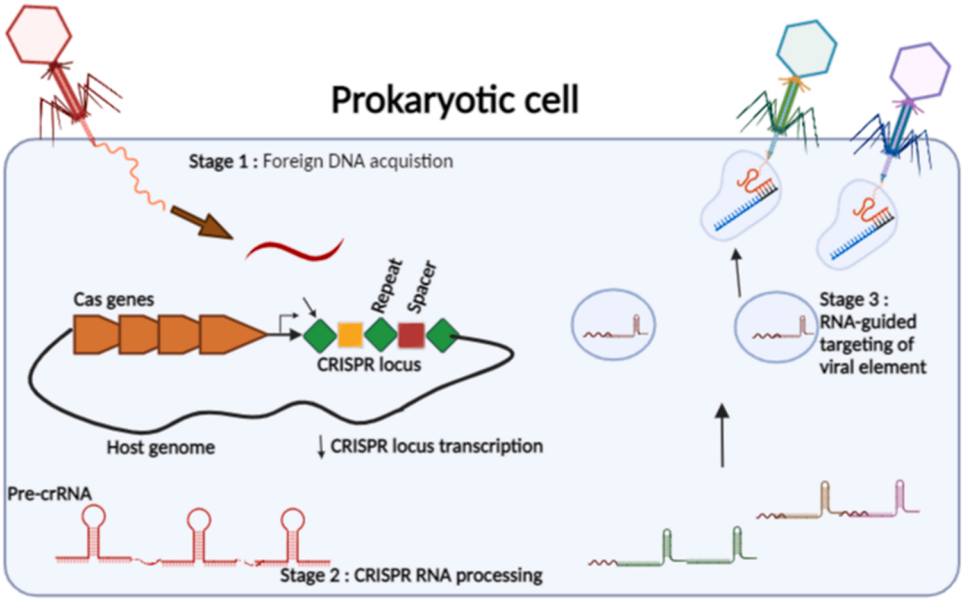


Fig. 1. CRISPR Cas system in prokaryotic cell showing the steps in mechanism.

CRISPR Associated Protein and CRISPR RNA (crRNA). CRISPR-Cas systems play three major roles; Adaptation, Biogenesis and Interference, while crRNA biogenesis is critical for the activation of the immune system because the cleavage products are needed for efficient assembly of the interference complex (Charpentier et al., 2015; Hille et al., 2018).

Adaptation: occurs in a fraction of foreign gene from an invading phage, also known as the protospacer; this spacer has been demonstrated experimentally in CRISPR subtypes: Type I-A (*Sulfolobus solfataricus*) (Erdmann and SGarrett, 2012), Type I-B (*Haloarcula hispanica*) (Li et al., 2014), Type I-E (*E. coli*) (Yosef et al., 2012), and Type II-A (*S. thermophilusit*) (Wei et al., 2015). It is picked and interfused into the CRISPR array as a new spacer; the two leading players in this phase are the cas1 and cas2 proteins. Both dimers form complexes for spacer acquisition. The cas1 protein has 2 enzymes nucleases and integrase, while cas2 has endoribonuclease, this is for bacteriophage with RNA genomes. (Fig. 2) shows this mechanism. Biogenesis or maturation of CRISPR RNAs (crRNAs) on the other hand explains how the CRISPR array is replicated and grown into mature crRNAs that consist of single spacers with flanking handles stemming from repeat. One of the spacer DNA strands is transcribed into mRNA consisting of both Crispr repeats and the viral genomes. In type I, Crispr repeats are looped and the mRNA is usually cut into smaller pieces (crRNA) by cas6e and cas6f enzymes. In type II, tracrRNA are bound to the CRISPR sequences on the mRNA cut by cas9 and RNase III enzymes, while in type III, cas6 homolog cut the mRNA directly. Finally, interference points to how the invaded foreign genetic piece is subverted (Zhang et al., 2012) by CRISPR-associated nucleases with the guidance of crRNA. In type I, the cas enzyme recognizes the PAM sequence and activates cas cascade enzymes (cas3) after binding resulting in a degraded viral genome. Type II, cas9 enzymes recognizes the PAM sequences after which it undergoes a double-strand break. In type III, the RNA sequence recognizes the complementary strand in the viral genome and binds to the cascade because there is no PAM in type III. The versatility of CRISPR based tools in modern applications such as nucleic acid detection, base editing, transcriptional regulation, gene editing, epigenome editing and easier operations, render huge impact/benefits in therapeutic applications.

The therapeutic potentials of CRISPR were demonstrated, especially in cas9, it shows to be an antimicrobial agent and can be used to specifically target antibiotic-resistant and highly virulent bacteria strains (Bikard et al., 2014; Citorik et al., 2014). Cas9 also holds prospects for treatments in HIV and Hepatitis B virus (Hu et al., 2014; Zhen et al., 2015). Applications in gene therapy were demonstrated by repairing the cftr gene in cultured cells from human cystic fibrosis patients (Schwank et al., 2013), curing dominant cataract disorder by fixing hereditary tyrosinemia in adult mice (Yin et al., 2014).

3. Classification of CRISPR Cas systems

CRISPR-Cas systems are classified into two classes (Class 1 and Class 2), these classes are based on the guidance in binding and target cleavage of different Cas proteins. The categories are also classified into six types based on the "signature genes" (Smargon et al., 2017) and forty-eight subtypes (Makarova et al., 2020a). Types I, III, and IV systems fall under the Class 1 systems based on their multi-subunit effector complexes, while Types II, V, and VI systems are grouped into Class 2 systems the single, large, multi-domain effectors (Makarova et al., 2015). These six classes of Cas systems use palpable effector complexes that initiate the destruction of foreign genetic DNA. For DNA, the CRISPR system that targets its strands are Types I, II and V, while RNA strands are targeted by type VI, whereas the type III system targets both DNA and RNA and the type IV systems have not yet been experimentally characterized (Koonin et al., 2017). Table 1 shows the bacteria species involved in Crispr Cas system. Class 1 systems, are frequently discovered in fungi, with a small percentage identified in bacteria (Makarova et al., 2017). Class 2 systems are commonly found in bacteria. Due to their simplicity and high efficiency, class 2 CRISPR-Cas systems have been widely utilized in genome editing and nucleic acid detection assays for human pathogens, especially CRISPR-Cas9, Cas12a and Cas13a. We will be discussing class 2 effectors in this article because they have more biotechnology applications and nucleic acid detection activity. As stated earlier, class 2 CRISPR systems consist of types II, V and VI. The distinguishing feature of these types is that their effector complexes consist of a single, large, multi-domain protein, such as Cas9 in type II. Since the 2015 classification, drastic expansion has been ongoing in a quest for

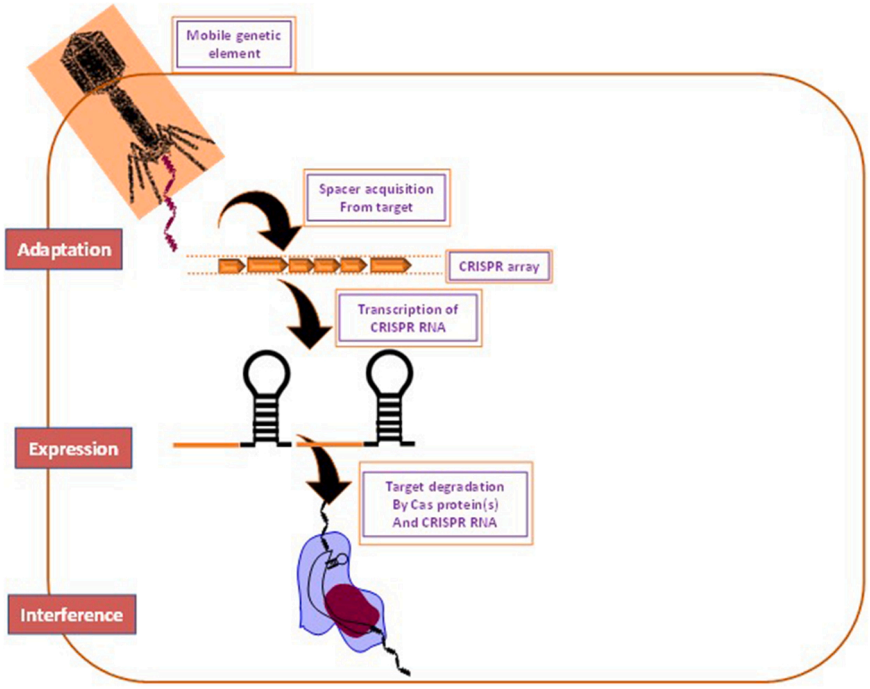


Fig. 2. Steps to CRISPR Cas immune system. [a]) Adaptation: The new genetic material is released into the CRISPR array by invading phage. [b]) Biogenesis: CRISPR array is replicated and CRISPR RNA is grown to maturity. [c]) Inference: destruction of invading genetic material by Cas proteins and CRISPR RNA.

Table 1
Types of Class 1 CRISPR Cas system in bacteria species.

CRISPR Cas systems	Bacteria Species	References
Type I	*E.coli, P.aeruginosa, M. xanthus, B. halodurans, C. concisus, C. curves, C. fetus, C. hominis, C. rectus, Y. pestis, Salmonella specie, E. amylovora, P. acnes*	(Brouns et al., 2008; Haurwitz et al., 2010; Makarova et al., 2011; Nam et al., 2012).
Type III	*P. furiosus, S. epidermidis, M. tuberculosis*	(Marraffini and LASontheimer, 2008;Hale et al., 2009;Abadia et al., 2010).
Type IV	*Thioalkalivibrio sp., Rhodococcus jostii, Thermoflexia bacterium*	(van der Ploeg, 2009; Gunderson and FFCianciotto, 2013;Louwen et al., 2013;Sampson et al., 2013; Weiss and DSampson, 2014).

(Adapted from (Makarova et al., 2020a))

potential new genome-editing tools with the help of computational discovery (Yan et al., 2019). From 2 types and 4 subtypes in 2015, class 2 has expanded to 3 types and 17 subtypes. This new discovery include multiple, diverse variants of type V and type VI systems. CRISPR Cas systems in class 2 can be seen in Table 2.

Type II CRISPR-Cas systems are the most well-known, with four subtypes distinguished by the Cas9 protein (Chylinski et al., 2014). The Cas9 protein is activated and guided to break the desired DNA sequence by a dual RNA, tracrRNA and crRNA. The repetitive portions of the pre-crRNA complement tracrRNA, whereas crRNA binds to complementary sequences in the target DNA. Cas9 recognizes PAM sequences (5-NGG-3) on target DNA through the crRNA-tracrRNA complex, and then the HNH and RuvC-like nuclease domains of Cas9 cleave double-stranded DNA and form blunt ends (Shmakov et al., 2015).

Table 2
CRISPR Cas systems in Class 2.

Types	Sub-types	Organisms and gene name
Type II	II-B	*Legionella pneumophila* str. Paris lpp0160–lpp0163
	II-A	*Streptococcus thermophilus* str0657–str0660
	II-C1	*Neisseria lactamica* 020–06 NLA_17660–NLA_17680
	II-C2	*Micrarchaeum acidiphilum* ARMAN-1 BK997_03320–BK997_03335
Type V	V-A	*Francisella* cf. *novicida* Fx1 FNFX1_1431–FNFX1_1428
	V-E	*Deltaproteobacteria bacterium* A2Z89_08250–A2Z89_08265
	V-B1	*Alicyclobacillus acidoterrestris* N007_06525–N007_06535
	V-B2	*Planctomycetes bacterium* RBG_13_46_10 A2167_01675–A2167_01685
	V-I	*Freshwater metagenome* (JGI) Ga0208225_100001036
	V-H	*Hypersaline lake sediment metagenome* (JGI) Ga0180438_100006283
	V-C	*Oleiphilus sp.* A3715_16885–A3715_16890
	V-D	*Bacterium* CG09_39_24 BK003_02070–BK003_02075
	V-F1	*Uncultured archaeon* NDOCEIEL_00008–NDOCEIEL_00011
	V-F1 (V-U3)	*Bacillus thuringiensis* HD-771 BTG_31928
	V-F2	*Uncultured archaeon* ICDLJNLD_00049–ICDLJNLD_00052
	V-U2	*Cyanothece sp.* PCC 8801 PCC8801_4127
	V-U4	*Rothia dentocariosa* M567 HMPREF0734_01291
	V-F3	*Candidatus Micrarchaeota* archaeon COU37_03050-COU37_03065
	V-U1	*Gordonia otitidis* GOOTI_RS19525
	V-G	*Hot springs metagenome* FLYL01000025.1 (182949.185252)
	V-K (V-U5)	*Cyanothece sp.* PCC 8801 PCC8801_2993–PCC8801_2997
Type VI	VI-A	*Leptotrichia shahii* B031_RS0110445
	VI-D	*Ruminococcus bicirculans* RBI_RS12820
	VI-C	*Fusobacterium perfoetens* T364_RS0105110
	VI-B1	*Prevotella buccae* HMPREF6485_RS00335–HMPREF6485_RS00340
	VI-B2	*Bergeyella zoohelcum* HMPREF9699_02005–HMPREF9699_02006

(Jinek et al., 2012) have developed a single-guide RNA (sgRNA) to replace the crRNA-tracrRNA dual RNA, which simplifies type II systems and makes CRISPR-Cas genome editing more attainable.(Table 3).

The domain structure of type V effector proteins distinguishes them from type II systems. The type II effectors (Cas9) have two nuclease domains, one for cleaving one strand of target DNA and the other for cleaving the other. The HNH nuclease is inserted inside the RuvC-like nuclease domain sequence (Chylinski et al., 2014). The type V effectors (Cas12), on the other hand, only have a RuvC-like domain that cleaves both strands (Strecker et al., 2019; Swarts and DCJinek, 2019).

Type VI systems, which have five derivative subtypes, are the most recently found. Type VI, like type V, does not require tracrRNA, and Cas13 is the distinctive protein with two HEPEN RNase domains (Anantharaman et al., 2013). CrRNA-Cas13 identifies proto-spacer flanking site (PFS) sequences and cleaves nearby non-target RNA in a nonspecific manner, destroying the target ssRNA with a blunt-ended break (O'Connell, 2019).

4. CRISPR Cas variants

All the Cas proteins are important effectors in CRISPR Cas systems but Cas9 and Cas12a have been manipulated to carry out different genome editing functions. CRISPR/Cas9 which is universally known for editing genome is acclimatized from the immune system of bacteria and archaea to protect them against bacteriophages and plasmid transformations. Cas9 a double RNA-guided nuclease that consists of both CRISPR RNA (crRNA) and tracrRNA has two cleaving domains RuvC and HNH, it has the Cas9 protein, guide RNA (gRNA) and an endonuclease (Jinek et al., 2012). It requires a 3′ NNGRRT PAMs sequence and targets

Table 3
Differences in the various CAS Variants.

CRISPR CAS VARIANTS	CLASS	TYPE	DOMAIN	FUNCTIONS
Cas9	Class 2	Type V	Cleaves to RuvC and HNH and creates double strand breaks	Used in genetic manipulation of genomes, especially in mammals
Cas12a	Class 2	Type V-A	Absence of tracrRNA	Creates and allow single-stranded enzyme (DNase) to cut the single-stranded DNA and used in genome editing. Cas12a has its own enzyme activity (RNase) and creates its own pre-crRNA array into individual crRNAs
Cas12b	Class 2	Type V	Presence of tracrRNA	Used in robust genome editing.
Cas13a	Class 2	Type VI	Cleaves to both targeted single-stranded RNA (ssRNA) and pre-crRNA	These are single Cas effectors with RNAses guided by crRNAs and resists RNA phages. Specific for detecting nucleic acids.
Cas 14	Class 2	Type V	Protospacer adjacent motif (PAM) is absent. Disallows cleavage to double-stranded DNA or single stranded RNA, allows cleavage to single-stranded DNA (ssDNA). Also possesses trans cleavage activity	Uniquely specific for nucleic acid detection

CRISPR Cas variants and their functions

sequences between 21 and 24 base pairs in length. Cas9 systems help genome editing by causing a double-strand break (DSB) at a target genomic locus, which is then repaired by the cell's DNA repair machinery. Non-homologous end joining (NHEJ) is used to join the ends of the DNA generated, which is known to be quite accurate (Bétermier et al., 2014) and can also cause mutations (insertion/deletion) at the DSB site (Lieber, 2010), especially when the nucleases in the cell have been active for a long time. Microhomology-mediated end joining (MMEJ), which introduces mutations at the target site, can also be caused by homology flanking the DSB. (Sinha et al., 2017). These mutations provide a disarray of protein-coding and other functional DNA sequences. Bacteria consisting of Cas9 systems include *Staphylococcus aureus* (SaCas9), *Streptococcus thermophilus* (St1Cas9), *Francisella novicida* (FnCas9), *Neisseria meningitidis* (NmCas9), *Campylobacter jejuni* (CjCas9) and *Staphylococcus aureus* (SaCas9) (Cebrian-Serrano and ADavies, 2017). *Staphylococcus aureus* (SaCas9) was one of the first bacteria in which CRISPR type II loci were identified (Deltcheva et al., 2011).

The CRISPR/Cas12a system (also referred to as Cpf1), was characterized in the genetic piece of *Prevotella* and *Francisella* (Schunder et al., 2013). The (gRNA) guider RNA particularly merge with the target genes to form three parts i.e (target nucleic acids, Cas12a protein and gRNA). Cas12a binds to its target sequence in association with a single RNA species, without requiring additional tracrRNA (Zetsche et al., 2015). Fluorescent signals can be monitored by the single-stranded DNA fluorescent probe produced by Cas12a protein where single-stranded DNase activity is cut (Chen et al., 2018a). Compared to the Cas9 family, the Cas12a-crRNA complex recognizes a T-rich PAM sequence that lies on the 5′ of its target sequence while in Cas9 systems, the G-rich PAM sequence lies in 3′ of its target (Zetsche et al., 2015). The T-rich PAM is known to ease genome engineering in organisms especially with AT-rich genomes. CRISPR/Cas12b (C2c1) is another RNA-guided nuclease method for editing the genome in the mammalian cells, which targets DNA. Comparing Cas 12a to Cas 12b, 12b is dual-RNA guided, that makes use of tracrRNA (Shmakov et al., 2015). Further research on Cas12 effectors has been done, identifying from microorganisms especially from databases of bacterial genomic, which includes Cas12c (Shmakov et al., 2015). Although, two Cas 12 effectors were found in the samples of metagenomics Cas12d (CasY) and Cas12e (CasX) (Burstein et al., 2017), and three sub-class of Cas12F (Cas14) (Chen et al., 2018c).

Cas 12e also known as Cas X is another RNA-DNA endonuclease that creates a non- blunt double-stranded DNA that breaks at complementary sequences to twenty nucleotide segments of its guide RNA. CRISPR-Cas proteins while analyzing the sequence of CasX unveils no distinct correspondence with each other, apart from the presence of a RuvC nuclease domain similar to that found in both Cas9 and Cas12a proteins respectively as well as transposases and recombinases. CasX also catalyzes planned, genome editing in human cells and site-specific genome repression in *E. coli*. Results in biochemistry analysis show that Cas X is a crossbreed enzyme that has both Cas 9 and Cas 12a elements making it the third effective tool in the CRISPR Cas family used in genetic manipulation.

Cas13a is an RNA-based tool that has been employed in various diagnostic approaches in detecting nucleic acids in pathogens and diseases. It has an RNA-guided enzyme (RNase) that binds the single-stranded RNA (ssRNA) target compared to Cas9 and Cas12a and pre-crRNA. After the cleavage of crRNA-targeted RNA is shown in vitro, Cas13a cleaves collateral RNA that has no complement to the designed crRNA (Abudayyeh et al., 2016). Due to its extensive use in RNA cleavage, it is explicitly designed for detection in nucleic acids. Although, precise and concise diagnostic tools in detecting a small number of nucleic acids should be employed even to the attomolar range. In 2017, SHERLOCK (Specific High-sensitivity Enzymatic Reporter Unlocking) an isothermal amplification technique was designed and combined with Cas 13a detection kit to achieve high sensitivity of RNA detection in nucleic acid sequences (Gootenberg et al., 2017a). Also, Cas 13a target sites could be signaled out and interpreted using an RNA reporter labeled with a quencher and fluorophore (East-Seletsky et al., 2016). After targets bind to Cas13a, collateral cleavage of the RNA reporter is released through the fluorophore from the quencher, which results in a fluorescent signal. CRISPR-Cas13a and Cas13b systems give resistance to RNA phages, and they are single-effector RNases guided by crRNA (Abudayyeh et al., 2016). Cas13a, b, c, and d have all been employed in mammalian cells to mediate targeted RNA knockdown (Cox et al., 2017).

Recently, (Pausch et al., 2020) discovered a new Crispr Cas member CasF (Cas12j) is a group of Biggie phage clade that is encoded in Cas protein (Al-Shayeb et al., 2020). CasF contains a C-terminal RuvC domain with homology remote also same as the TnpB nuclease superfamily where type V CRISPR Cas proteins are emanated from. Cas F has three orthologs namely, Cas F-1, Cas F − 2 and Cas F-3, respectively, and is examined from metagenomics assemblies. Compared to CasF-1, CasF-2 and CasF-3 were more active in vitro, and within the RuvC active sites, the non–target strand (NTS) cleaved faster than the target strand (TS). CasF is an original CRISPR effector that allows the cleaving of crRNA-complementary DNA just like other phages and its works effectively in its natural habitat as a phage protein. This proves that this CasF is more succinct than other CRISPR systems. Furthermore, CasF does not cleave ssRNA in cis or in trans but single-stranded DNA, which shows CasF can also target intermediates of ssDNA MGEs or ssDNA.

Cas 14, grouped under Class 2 Cas effectors, has a rigid protospacer adjacent motif (PAM) that binds to DNA. PAM binding is innate into the Cas protein, unlike RNA-guided binding that is complementary to DNA target. Cas14 does not cleave to double-stranded DNA or single-stranded RNA, it only cleaved to single-stranded DNA (ssDNA). Cas14 is solely designed for detection in sequences. Hence, it is specific for nucleic acid detection.

5. CRISPR technology in diagnostics and nucleic acid detection

CRISPR-based diagnostic approaches work by finding a specific sequence associated with a disease and then cleaving it to produce a readable signal, based on the concept that nucleic acids are effective biomarkers for diseases. Bacterial and viral sequences or oncogenic mutations sequences originating from infectious agent are examples of target sequences (Katalani et al., 2020). The purpose of CRISPR systems is to identify specific pathogens and correct disease-causing alleles by editing specific DNA sequences at precise chromosome regions (Uppada et al., 2018). The CRISPR system's diverse features have led to several diagnostic tools. While some tests rely on both the identification and cleavage of the target, others rely solely on the identification of the target's guide RNA and Cas protein (Uppada et al., 2018). CRISPR-based diagnostic tools have a lot of potential for applications in different domains because of their swift, sensitive, specific, accurate, affordable, and dependable qualities (Khambhati et al., 2019; Li et al., 2019b). They are capable of detecting infections during an outbreak, diagnosing cancer, identifying single-nucleotide polymorphisms (SNPs), and detecting genetic diseases (Khambhati et al., 2019). CRISPR system was generally known for its powerful genomic manipulations in bacterial cells in 2013 (Cong et al., 2013). The applications of CRISPR Cas systems in detecting nucleic acid are further explained in this manuscript. Concise and precise nucleic acid tests are essential for clinical diagnosis and human health. With the help of CRISPR Cas9, strains of Zika virus were easily differentiated through nucleic acid detection (Myhrvold et al., 2018a).

NASBACC (NASBA-CRISPR) was refined by merging an RNA amplification technique known as NASBA (Nucleic Acid Sequence-based Amplification) and CRISPR system (Pardee et al., 2016). Also, Cas13a is known in vitro as a very concise diagnostic tool used to detect nucleic acid (Gootenberg et al., 2017a). As soon as non-targeted RNAs are recognized by Cas13a-crRNA complex upon cleavage, the activated Cas13a cut the closest one. Unless the RNA is cleaved, all fluorescent RNA must emit fluorescence. In contrast, an RNA reporter quenches

those that do not emit fluorescence. This system is then effective through a technique "isothermal amplification" with recombinase polymerase amplification (for amplifying signals) and T7 transcription (for detecting DNA signals) (Piepenburg et al., 2006), generating a system called "SPECIFIC HIGH-SENSITIVITY ENZYMATIC REPORTER UNLOCKING" (SHERLOCK). This technique can detect DNA and RNA in their smallest specificity and sensitivity. In 2017, a research group developed HOLMES (One-Hour Low-cost Multipurpose Highly Efficient System), a system used in detecting nucleic acid. Using Cas12b and Loop-Mediated Isothermal Amplification (LAMP), HOLMESv2 was used easily in the detection of nucleic acids in 2018 (Li et al., 2018a). Subsequently, DNA endonuclease-targeted CRISPR trans reporter (DETECTR) was designed with the combination of CRISPR tools (Chen et al., 2018b), HUDSON (Heating un-extracted diagnostic samples to obliterate nucleases) (Myhrvold et al., 2018b) and SHERLOCKV2 (Gootenberg et al., 2018b) are the three novel techniques used in detecting nucleic acid. There is not much difference between HUDSON system and SHERLOCK since they carry out similar basis. Before SHERLOCK procedures is done, the viral particles found in the bodily fluid will be lysed first and the ribonucleases will be inactivated with the use of heat and chemicals. The avail of HUDSON over other methods is that it is time effective and extraction of nucleic acid is not necessary (Myhrvold et al., 2018b). DETECTR as a method is formed on activated target on Cas12a, without a specific ssDNase cleavage activity. HPV64 and HPV16 was identified successfully with high sensitivity and specificity (Chen et al., 2018c). (Fig. 3) shows a schematic representation of HUDSON, SHERLOCK and DETECTR techniques. Multiplex nucleic acid detection can be achieved with SHERLOCKv2 even comprising of Csm6, Cas13 and Cas12a and extremely high sensitivity.

Recently, SHERLOCK has been refined in detecting SARSCoV-2 leading to STOP (Sherlock Testing in One Pot) method, which can be used directly in POC applications (Kellner et al., 2019b). This improved SHERLOCK application takes 40–70 min with visual read-outs resulting in just 3–5 steps. Aside from the fact that RNA extraction is not required, this method has some advantages over the previous one, like using nasal and saliva swabs as samples. It is rapid and accurate in detection. It utilizes just one temperature and a fluid handling step. This method does not require adept personnel and can be performed ordinarily by lay users, and another pronounced advantage is almost no equipment required. Furthermore, a new SARSCoV2 detection protocol-based platform CDetection (Cas12bmediated DNA detection) (Teng et al., 2019) was used recently. This was carried out by combining nucleic acid amplification methods with CDetection and sample treatments protocols to achieve an incorporated viral nucleic-acid detection platform—CASdetec also known as CRISPR-assisted detection. This platform has a detection limit of 1×10^4 copies/mL for SARSCoV2 pseudo virus with no observed cross-reactivity. Coinciding with the general SARSCoV2 genome sequence (Paden et al., 2020), 7 sgRNAs around the RdRp locus was designed, according to World Health Organization (WHO) suggestions. SARS-CoV-2 and SARS-CoV are highly similar. Therefore, initial experiments were carried out on both viruses' plasmids. The study shows fluorescence kinetics proved sgRNA3 was dissimilar and distinguishable between the viruses generating disparate florescence signals.

Generally, recent studies have proved that 16% of world-known malignant tumors align with infectious agents (Ahmad et al., 2018). Most of these viral-related cancers are associated with Human papillomavirus (HPV), Hepatitis B virus (HBV), Hepatitis C virus (HCV) and *Helicobacter Pylori,* also in some cases, coinfection may occur (Ahmad et al., 2018). CRISPR/Cas9 is known as a novel technology for both viral applications and treatments in eradicating cancer initiation in the host cell (Kennedy et al., 2015). Human papillomavirus (HPV) is a viral infection of the reproductive tract, affecting women and men and later progresses to cancer (Organization, 2017). Using a technique known as Specific High-Sensitivity Enzymatic Reported Unlocking (SHERLOCK), combined with CRISPR/Cas13a (C2c2) and isothermal amplification (Gootenberg et al., 2017c), results in the detection of Zika virus (ZIKV) and Dengue virus (DENV) which has similar symptoms, gene mutations and are noninfectious disease. DETCTR and SHERLOCK illustrate the wide benefits of CRISPR/Cas system, and how they can be employed for diagnosis (Chertow, 2018). The operations of CRISPR/Cas system in virology is not just limited to its curative activity. DNA endonuclease-targeted CRISPR trans reporter (DETCTR) was developed and combined with CRISPR Cas proteins which enable accurate and precise detection of HPV, providing an easy platform for molecular diagnostics (Chen et al., 2018a). The characteristics and applications of CRISPR-based diagnostics technologies is shown in Table 4.

Hepatitis B virus is a major global health problem because chronic HBV infection leads to liver cirrhosis, which leads to cancer (Liu et al., 2015). Viral replication can be inhibited by antiviral therapies using nucleoside analogs, but does not destroy the existing HBV DNA that has been integrated (Li et al., 2017). Some novel contagious diseases, such as Zika virus (ZIKV) infections as created awareness of the need to build accurate detection kits for clinical practice in some undeveloped areas. Zika virus has some strain-specific PAM sites, such as African and American Zika variants. Hence, scientists designed a novel technique to distinguish between ZIKV strains based on the sequence-specific nuclease activity of CRISPR/Cas9 with single-base discrimination. The presence of an "NGG" PAM is necessary for the specific cleavage by Cas9 (Pardee et al., 2016).

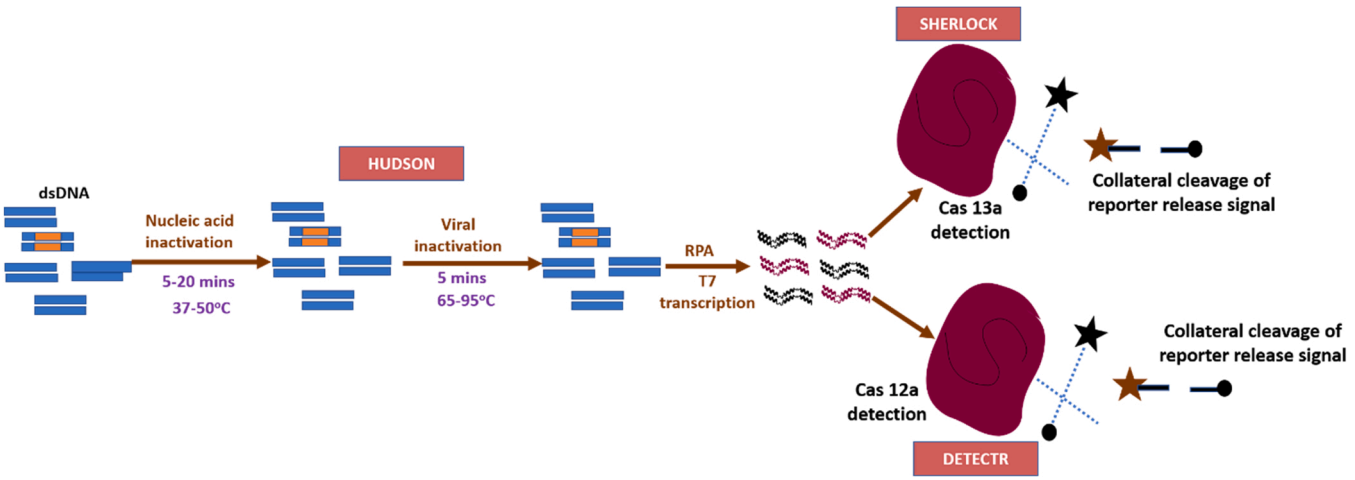


Fig. 3. Schematic diagram showing SHERLOCK, HUDSON and DETECTR techniques.

Table 4
Characteristics and application of CRISPR based diagnostics technologies.

Type of CRISPR system	Method	Effectors	Proteins	Amplification	Detection	Targeted Pathogens	Targeted types	Time	Reference
Type V	DETECTR	LbCas 12a	Cas 12a	RPA	Flouresence	HPV 16/18	DNA	2 h	(Chen et al., 2017)
Type VI	SHERLOCK	LwCas13a	Cas 13a	RPA	Flouresence	Viruses/ bacteria	DNA/RNA	2–5 h	(Gootenberg et al., 2017b)
Type III	SHERLOCK v2	LwCas 13a	Csm6	RPA	Lateral flow assay	Viruses/ bacteria	DNA/RNA	0.5–43 h	(Li et al., 2018e)
Type V		CcaCas13b	Cas 13		Flouresence				
Type VI		PsmCas13b	Cas 12a						
Type III, VI	HUDSON+ SHERLOCK	LwCas13a	Cas 13a	RPA	Flouresence Colorimetry Lateral flow	Viruses/ bacteria	RNA/DNA	<2 h	(Myhrvold et al., 2018b)
Type V	HOLMES	LbCas 12a	Cas 12a	PCR	Flouresence		DNA/RNA	~1 h	(Li et al., 2018c)
Type V	HOLMESv2	LbCas 12a	Cas 12a	LAMP	Flouresence		DNA/RNA	~1 h	(Li et al., 2019a)
Type V	CDetection	AaCas 12b	Cas 12a	RPA	Flouresence	HPV	DNA	~3 h	(Teng et al., 2019)

LwaCas13a:Leptotrichia wadeii Cas13a
CcaCas13b:Capnocytophaga canimorsus Cc5 Cas13b
PsmCas13b:*Prevotella species*. MA2016 Cas13b
LbCas12a:Lachnospiraceae bacteruim ND2006 Cas12a
AaCas12b: *Alicyclobacillus acidiphilus* NBRC 100859 AaCas12b
RPA: Recombinase polymerase amplification.

5.1. Specific high-sensitivity enzymatic reporter unlocking (SHERLOCK)

SHERLOCK, a diagnostic tool based on type VI of CRISPR Cas systems (Gootenberg et al., 2017a; Kellner et al., 2019a) works on the same principles as DETECTR; however it relies on Cas13 nuclease activity from *Leptotrichia wadei*. Cas13 recognizes and cleaves only to RNA unlike Cas12 cleaves to DNA. Recognition of DNA targets is possible due to in vitro transcription of the isolate. RPA can enrich target molecules and improve sensitivity by using isothermal amplification. Cas13 protein crRNA and fluorescent RNA probes are combined with the amplified RNA fragments. If the target molecules are present in the sample, Cas13 detects them via crRNA and cleaves fluorescent RNA probes indiscriminately (via collateral activity), destroying the fluorophore-quencher association. The amount of the target in the biological sample is thus determined by the presence and intensity of the fluorescent signal. With attomolar sensitivity, SHERLOCK detects Zika virus, dengue virus, numerous microbial pathogens, and single nucleotide polymorphisms (SNPs) in DNA (Gootenberg et al., 2017a). The initial SHERLOCK system had a key flaw in that it was qualitative rather than quantitative; however, scientists presented the second system, SHERLOCKv2, a year later (Li et al., 2018f). SHERLOCKv2 increased sensitivity by 3.5-fold by combining Cas13a with Csm6, a type III CRISPR effector nuclease that may connect its reporter signal with Cas13a for signal enhancement (Kazlauskiene et al., 2017).

5.2. Heating unextracted diagnostic samples to obliterate nucleases (HUDSON)

Heating unextracted diagnostic samples to obliterate nucleases (HUDSON) was created by (Myhrvold et al., 2018a) to identify viral nucleic acid directly from the physiological fluid using SHERLOCK. HUDSON uses heat and chemical reduction to lyse viral particles and inactivate high amounts of ribonucleases present in human fluids (Weickmann and JGlitz, 1982). According to this study, HUDSON treated urine or saliva could be introduced merely to RPA reaction mixes without dilution or purification. To avoid solidification throughout the technique, blood samples were diluted 1:3 in phosphate-buffered saline (PBS) without hindering further amplification or detection. Despite lower viral titers than those in serum, HUDSON and SHERLOCK enabled sensitive detection of free ZIKV nucleic acid spikes into a total turnaround time of 1 h for saliva (Andries et al., 2015). DENV was detected via lateral-flow strips and a colorimetric readout.

5.3. One-hour low-cost multipurpose highly efficient system (HOLMES)

Cas12a, which belongs to the class 2 type V-A CRISPR-Cas system (Zetsche et al., 2015), was discovered in 2018 to perform collateral cleavage on non-targeted ssDNA when the Cas12a/crRNA/target DNA ternary complex was formed (Li et al., 2018b). Scientists developed HOLMES (an one-HOur Low-cost Multipurpose extremely Efficient System), which could be used for swift detection of target DNA and RNA, using a quenched fluorescent ssDNA reporter as the probe. In HOLMES, if a target DNA exists in the reaction system, the Cas12a/crRNA binary complex forms a ternary complex with the target DNA, which will then trans-cleave non-targeted ssDNA reporter in the system illuminating the fluorescence. Ten Cas12a proteins were examined to find the most effective Cas12a protein for HOLMES (i.e. with high signal-to-noise ratios), *Francisella tularensis* Cas12a (FnCas12a), *Oribacterium sp.* NK2B42 Cas12a (OsCas12a), *Lachnospiraceae bacterium* ND2006 Cas12a (LbCas12a) and *Lachnospiraceae bacterium* NC2008 Cas12a (Lb5Cas12a) demonstrated good results as well as ssDNA trans-cleavage activity. (Fig. 4) illustrates the HOLMES method.

HOLMES can also identify DNA viruses (such as the pseudorabies virus (PRV)) and RNA viruses (such as the Japanese encephalitis virus) (JEV). HOLMES' great specificity also allowed it to differentiate between viral strains. The gE46 site, for example, was able to distinguish between the PRV Ra classical strain, the cmz variation strain, and the Bartha-K61 vaccine strain (Li et al., 2018d). In the HOLMES test, PCR amplification was paired to increase sensitivity. Isothermal amplification methods such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) can be used. HOLMES, similar to SHERLOCK, does not require expensive reagents or specific instruments, making it a low-cost and easy-to-use nucleic acid detection method.

5.4. DNA endonuclease-targeted CRISPR trans reporter (DETECTR)

In 2017, Jennifer Doudna's lab (Chen et al., 2018a) introduced the DNA endonuclease-targeted CRISPR trans reporter, a CRISPR-Cas diagnostic tool (DETECTR). This approach relies on the Cas12a protein's collateral activity, which is initiated once Cas12a recognizes the target RNA. After identifying target RNA, the authors showed that theCas12a protein from the *Lachnospiraceae* bacterium strain ND2006 (LbCas12a) has nonspecific collateral activity and destroys all nearby DNA molecules. When the Cas12a protein is combined with single-stranded DNA reporters (probes) and mixed with the biological sample, the

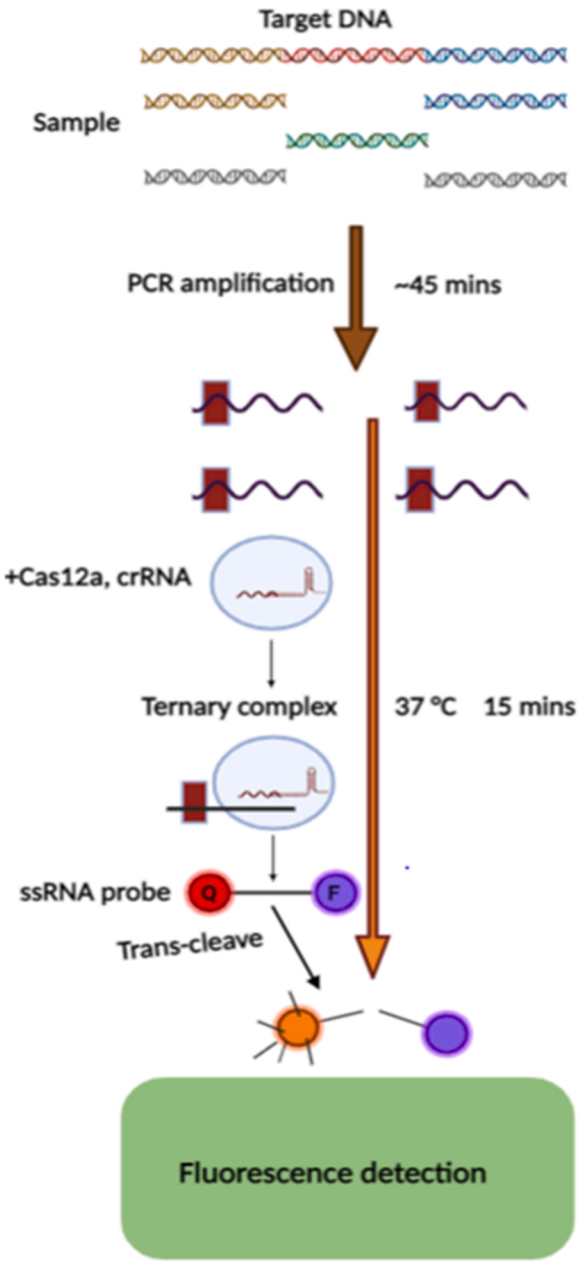


Fig. 4. Schematic illustration showing the HOLMES method.

crRNA-dependent detection of pathogenic nucleic acids by Cas12a activates collateral activity that destroys the DNA probes. DNA probes are similar to TaqMan probes in that one end of the reporter is attached to a fluorophore and the other end is attached to a quencher. Fluorophores are released as the DNA probes degrade, resulting in a steady and robust fluorescent signal that may be measured by a fluorimeter. DETECTR was also combined with an isothermal pre-amplification step to enhance target sequences (RPA). RPA improves the diagnostic test's analytical sensitivity and reduces complex and expensive equipment requirements. Other orthologous proteins from various organisms, such as AsCas12a (*Acidaminococcus sp.*), FnCas12a (*Francisella novicida*), and AaCas12b (*Alicyclobacillus acidoterrestris*) (Makarova et al., 2020b), exhibit collateral activity and can be used to develop diagnostic platforms in the same way that DETECTR can. DETECTR has been utilized to identify HPV and distinguish between HPV16 and HPV18, the most pro-oncogenic strains of HPV (Tsou et al., 2019). Interestingly, the DETECTR analysis takes only an hour to complete (Chen et al., 2018a).

5.5. Cas12b-mediated DNA detection (CDETECTION)

Using its potential to trans-cleave single-stranded DNA (ssDNA) reporter, the Cas12a-DETECTR technology can directly detect double-stranded DNA (dsDNA) samples (Gootenberg et al., 2018a). Even though the discovery that AaCas12b (Teng et al., 2018) performs DNA-recognition-triggered collateral cleavage has striking similarities to Cas12a orthologues, the AaCas12b effector's strong cleavage activity has provided CDetection with increased signal amplification and enhanced sensitivity. CDetection, in particular, has a minimal LOD of 0.1 aM, as demonstrated by analyses on HPV dsDNA diluted with human genomic DNA. Furthermore, when paired with tgRNA, a designed gRNA with a mismatch in the spacer sequence that allows single-nucleotide resolution screening of targets, CDetection was able to distinguish between 3232 A > G SNP sites and wild-type alleles with allelic fractions as low as 1%. This study found Cas12b's unique capability, pointing to the possibility of effective and diverse nucleic acid detection methods. (Fig. 5) shows Cas12-based diagnostic tools in HOLMESv2 and CDetection.

6. Advantages of CRISPR Cas systems over other Amplification methods

Early diagnosis is very effective for controlling the spread of disease/infection. Hence, this is also applicable to nucleic acid detection, and immunoassays nucleic acid detection is the most popular testing methods. However, detection in blood samples for immunoassays is usually hectic, especially when there are no signs and symptoms. Due to other similar coronaviruses, cross-reaction may affect the efficiency of immunoassays (Lee et al., 2020). Furthermore, while diagnosing COVID-19, immunoassays cannot be used as the basic method. The general standard of detecting of SARS-CoV-2 is the extraction of samples from the throat and nasal swab using qRT-PCR (Wu et al., 2020), but this requires more heating and cold process with the use of costly equipment. Due to the complexity of operations, qRT-PCR stands as a barrier to most health sectors, especially in underdeveloped countries.(Fig. 6).

Generally, nucleic acid detection methods/techniques should be easy to use, portable, sensitive, specific, cost-effective and rapid. Mainly, nucleic acid detection methods in clinical practice include amplification methods like recombinase polymerase amplification (RPA), nucleic acid sequence-based amplification (NASBA), quantitative real-time PCR (qPCR), loop-mediated isothermal amplification (LAMP), fluorescence in situ hybridization (FISH) e.t.c but these methods still have limitations. FISH is time-consuming because of the lengthened heat treatment required for its probe hybridization which causes a holdback in sensitivity and/or specificity. With CRISPR Cas12a protein, nucleic acid detection in SARS-CoV-2 is time efficient and results can be seen with the naked eye in a smartphone. (Myhrvold et al., 2018a) reported that use of Cas13-based nucleic acid detection platform SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) can differentiate between viruses with similar symptoms, like zika virus ZIKV and dengue virus DENV respectively (Faye et al., 2008). Also, SHERLOCK assay was used to classify various Zika and dengue viral species and strains.

7. Challenges and open research issues

In this review, we discussed various variants of CRISPR Cas systems, detailed applications of CRISPR Cas systems were highlighted in detecting nucleic acids, the role of CRISPR Cas system in detecting nucleic acids, and stated the advantages of CRISPR systems over other amplification methods. As we have discussed in this paper, CRISPR Cas-based systems combined with other isothermal methods for detection of nucleic acids are more swift, portable, accurate, inexpensive and a time-effective tool than PCR or RT-qPCR techniques which is time-consuming and requires more labor (Varlamov et al., 2020). Moreover, some new Cas variants such as Cas Y (Cas 12d), Cas X(Cas 12e), Cas F (Cas 12j) and

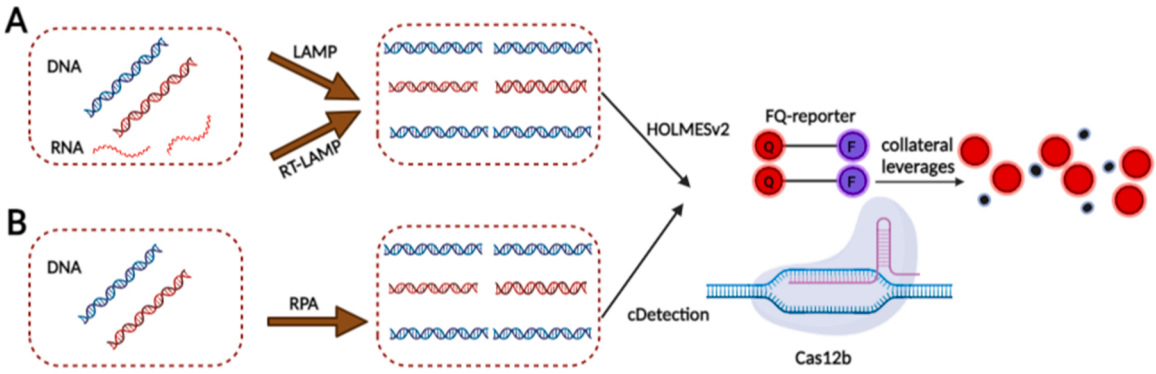


Fig. 5. Standard illustrations of Cas12-based diagnostic tools in HOLMESv2 and CDetection. (A) HOLMESv2 enabling isothermal detection by the integration of AaCas12b system with LAMP. (B) CDetection enabling ultrasensitive DNA detection by the integration of AaCas12b system with RPA.

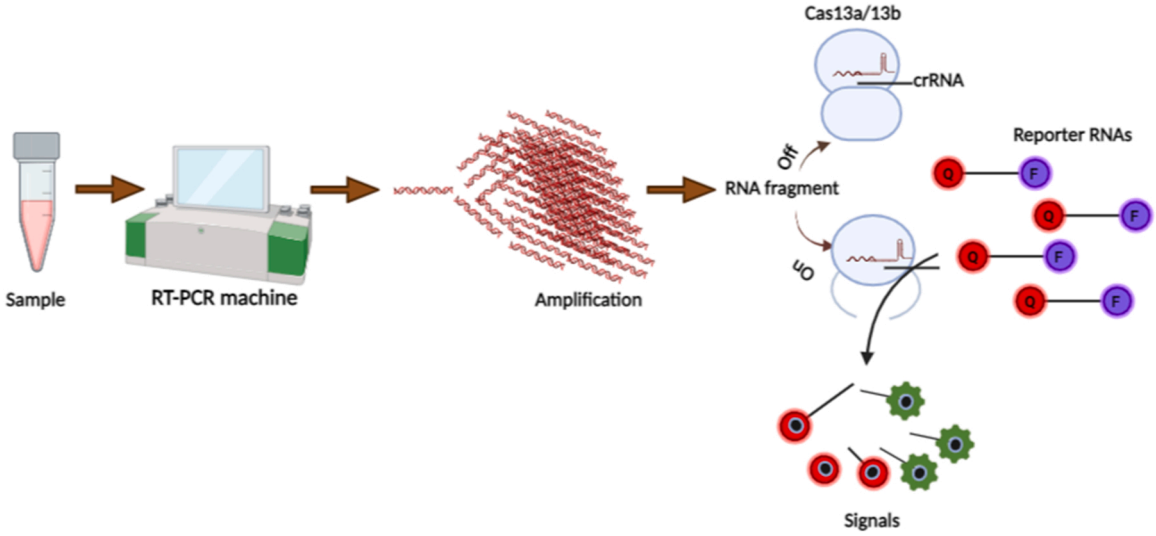


Fig. 6. Schematic illustration of a rapid molecular diagnostic technique using CRISPR Cas based technology using Cas13a and Cas13b proteins.

Cas 14 were discovered, which will be more helpful in application in detecting the specific double- or single- stranded nucleic acid with their less molecular amount and more active. (Burstein et al., 2017; Koonin et al., 2017; Al-Shayeb et al., 2020).

Scientists and medical personnel could use CRISPR Cas systems in combination with isothermal approaches to diagnose viral and bacterial infections and cancer mutations. Although the CRISPR Cas system is a promising diagnostic tool for nucleic acid detection, it is not without its limitations. Quantitative analysis is highly significant in molecular diagnosis. The concentration level of nucleic acids found in liquid biopsies varies with the disease's development in diagnosing various diseases, and quantitative analysis may be required for quick diagnosis. Without on-site calibration, quantifying the results is incredibly challenging. CRISPR-based detection methods such as SHERLOCK, DETECTR, and others are only qualitative and cannot quantify the number of nucleic acids present.

8. Conclusion

Tremendous headway has been made in applying and manipulating various CRISPR-Cas systems which have changed the molecular biology field, particularly in human cells. Hence, other CRISPR-associated nuclease variants are being discovered beside the generally known Cas9 system, providing potential improvements in efficacy, accuracy and delivery. Due to Cas 12 small molecular size, it is best for viral packaging compared to Cas9, it is greatly employed for multiplex genome editing because multiple guide RNAs can be easily expressed as a single transcript and subsequently processed into individual guide RNAs by Cas12a itself. In this review, the most profound benefit of CRISPR-based nucleic acid detection tools is discussed and programmed for diverse applications that work quickly due to their accuracy, speed and sensitivity. Notwithstanding, with the emergence of these modernistic applications of CRISPR and exploration of more ostensive Cas orthologs, this technology has helped to solve many diagnostic problems that serve as a threat to human lives. Although, more researches are ongoing as scientists globally explore more CRISPR Cas proteins for development in mammalian cells.

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Consent for publication

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Data Availability

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Competing interest

The authors declare no conflict of interest.

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