Review Article
Open Access
Life Sciences, Medicine and Biomedicine

CRISPR: An overview of CRISPR-Cas systems and their diagnostic methods for pathogen detection

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Received 23 October 2025; Revised 26 March 2026; Accepted 7 April 2026; Published online 30 June 2026

ABSTRACT

Early and rapid diagnosis of pathogens is crucial for preventing and controlling epidemic diseases. While traditional microbial detection methods serve their purpose, they often have limitations such as low sensitivity and specificity. In contrast, the CRISPR-Cas system is known for its high specificity, high sensitivity and robustness which offers a promising alternative for pathogen detection. This review provides an update on the CRISPR-Cas system, covering the history and classification of CRISPR-Cas systems. It also outlines the diverse CRISPR-based strategies and summarizes recent advances in nucleic acid–based diagnostic methods for pathogenic detection, thus broadening the understanding of CRISPR's diagnostic potential. The review also focuses on the different CRISPR-Cas systems-based detection of pathogenic microorganisms and recent advances of each nucleic acid-based detection method. This serves to enhance the understanding of the broader implications of the CRISPR system. Beyond the current state of CRISPR-Cas system applications, some of the challenges associated with CRISPR-Cas system usage are addressed, offering insights that could guide innovative solutions and advancements. This review looks ahead to future research perspectives by using CRISPR-Cas technology as a novel platform of next-generation diagnostics. By integrating the current applications, emerging challenges and diagnostic strategies, this review provides a comprehensive overview of CRISPR-Cas systems. Additionally, this review sets itself apart by emphasizing system-specific CRISPR-Cas strategies that support the advancement of next-generation diagnostic platforms.

Keywords: *CRISPR-Cas; nucleic acid-based detection and next-generation diagnostics*

INTRODUCTION

According to the World Health Organization (WHO), antibiotic resistance has been recognized as a major threat to public health, with an estimated 700,000 deaths annually attributed to treatment-resistant infections (United Nations, 2019). This phenomenon is fuelled by various factors, including the overuse and misuse of antibiotics in human and animal health, as well as in agriculture. The inappropriate use of antibiotics contributes to the spread of resistant bacteria, which can render common antibiotics ineffective in treating infections. For example, in 2023, a study published by The Lancet Infectious Diseases reported that over 50% of *Klebsiella pneumoniae* isolates in some regions are resistant to carbapenems, a last-resort antibiotic class, due to enzymes like *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo-ß-lactamase (NDM). These infections have high mortality rates (40-50%) and limited treatment options, often requiring toxic alternatives like colistin (World Health Organization, 2017). The spread of carbapenem-resistant *Enterobacterales* (CRE) is fuelled by inappropriate antibiotic use (Naghavi et al., 2024). The alarming statistics

https://doi.org/10.28916/lsmb.10.1.2026.232

context, in the fight against antibiotic resistance, the CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated proteins) system has emerged as a potential diagnostic tool. A recent study reported by Ahmed et al. (2024), indicates that CRISPR offers the potential to combat antibiotic resistance in bacteria by precisely targeting and modifying specific genes. This strategy could pave the way for new antibiotic treatments or enhance the effectiveness of existing ones (Ahmed et al., 2024). Information about the CRISPR-Cas system, initially discovered as a bacterial immune system, has advanced significantly since 2015, leading to an updated evolution-based classification of CRISPR-Cas systems and cas genes. Major developments in the discovery of novel types and subtypes of CRISPR-Cas systems, their diverse mechanisms of action, and the identification of numerous proteins that can inhibit CRISPR-Cas activity were reported (Bondy-Denomy et al., 2013). These advancements have expanded the applications of CRISPR technology in genome editing, biotechnology and therapeutics, highlighting the complexity and versatility of these systems in microbial defense and genetic manipulation (Makarova et al., 2011, 2018).

As the functions and mechanisms of CRISPR systems are gradually revealed, the applications of CRISPR-Cas systems are increasingly exploited across a myriad of domains. In healthcare, the precision and efficiency of CRISPR-based gene editing hold immense potential for treating genetic disorders, cancer and prevention of infectious diseases (Liu et al., 2022). Importantly, the same molecular precision that underpins CRISPR's gene-editing capabilities has also enabled the development of rapid, sensitive, and cost-effective diagnostic platforms (Ding et al., 2020), revolutionizing disease surveillance and outbreak management as well as controlling the spread of antimicrobial resistance. Furthermore, the versatility of CRISPR technology extends to basic research, where it is employed, for example, to unravel the intricacies of gene regulation, cellular pathways and microbial interactions (Sardar et al., 2024). With ongoing research, CRISPR-Cas technology continues to advance, offering innovative solutions to challenges in healthcare.

This review comprehensively explores the multifaceted landscape of CRISPR-Cas systems and their pivotal role in pathogen detection. Spanning from historical roots to contemporary classifications, it delves into the intricate mechanisms of CRISPR-Cas and its implications for preventing the spread of antibiotic resistance and pathogenicity, particularly among microbes mentioned on the WHO's list of global priority pathogens. Moreover, the review meticulously examines the diverse array of detection methods harnessing various CRISPR-Cas types, including Cas9, Cas12, Cas13 and Cas14, shedding light on their respective applications and potential for next-generation diagnostics. Additionally, by addressing challenges and outlining future perspectives, this review navigates the trajectory of CRISPR-Cas detection systems towards advancing the frontiers of molecular diagnostics.

History of CRISPR-Cas system

The development and implementation of the CRISPR-Cas system have been a significant milestone in understanding and manipulating genetic material, leading to numerous applications in various areas of research, medicine and biotechnology. The history of the CRISPR-Cas system spans several decades and involves numerous scientific discoveries and breakthroughs.

The discovery of this system began in the late 1980s, when researchers identified the gene of the protein responsible for isozyme conversion of alkaline phosphatase (*iap*) in the bacterium *Escherichia coli* and noticed that certain DNA sequences in the genome were repeated at regular intervals, with short "spacer" sequences in between (Ishino et al., 2018; Shmakova et al., 2022). For nearly two decades, the function of these repetitive sequences (CRISPR) remained a mystery until 2005, whereby Mojica et al., (2005) reported that CRISPR might be involved in an immune system-like defense mechanism in bacteria. The authors speculated that CRISPR might act as a form of acquired immunity, to protect bacteria from viral infections (Mojica et al., 2005). In 2007, Barrangou et al., working with virulent bacteriophages isolated from industrial yogurt samples, demonstrated that CRISPR sequences were indeed part of an adaptive immune system in bacteria whereby bacteria could incorporate small segments of viral DNA into their CRISPR arrays, which then acted as a molecular memory to defend against future viral infections (Barrangou et al., 2007).

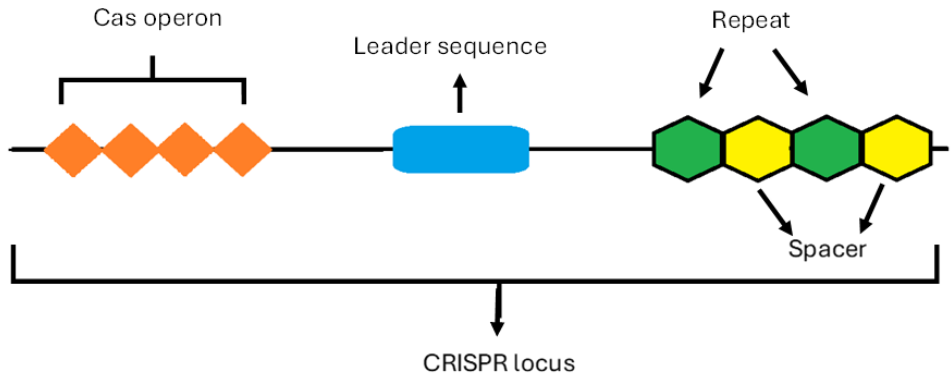
Such clusters of regularly interspaced short palindromic repeats were later found in other bacterial and archaeal genomes, including *Streptococcus thermophilus*, *Mycobacterium tuberculosis, Shigella dysenteriae, Salmonella typhimurium*, *Streptococcus pyogenes* and *cyanobacteria* such as *Anabaena sp.* (Javed et al., 2018; Shmakova et al., 2022). The term CRISPR was first introduced in 2002 and the first CRISPR-associated (Cas) genes were described in the same year. While these early discoveries shed light on the existence and potential function of CRISPR, the true breakthrough came in 2012 when Jinek et al., independently demonstrated that the CRISPR system could be harnessed as a precise gene-editing tool (Jinek et al., 2012). They focused on a specific component of the CRISPR system, a protein called Cas9, along with the realization that Cas9 could be programmed with a guide RNA molecule to target and cut specific DNA sequences with incredible precision (Gostimskaya, 2022). This breakthrough discovery marked the beginning of the CRISPR-Cas9 revolution. The CRISPR-Cas9 system's simplicity and effectiveness as a gene-editing tool sparked an explosion of research and applications in various fields. Scientists quickly adopted CRISPR-Cas9 for a wide range of genetic manipulations in diverse organisms, from simple bacteria to complex human cells and even entire organisms. In this review, we will explore the historical development of the CRISPR-Cas system, its classification, the most recent discoveries of CRISPR-Cas systems and its classification.

Classification of CRISPR-Cas system

The CRISPR-Cas locus typically consists of a CRISPR array along with adjacent Cas genes, forming operons that include leader sequence and direct repeats of approximately 25 to 48 base pairs (bp) (Shmakov et al., 2020; Wang et al., 2020a). These units are interspersed with distinct inserts of similar length called spacers, ranging from 26 to 72 bp, some of which share similarities with segments of virus or plasmid genomes (Butiuc-Keul et al., 2022; Hille & Charpentier, 2016; Koonin & Makarova, 2019; Shmakov et al., 2020). Figure 1 showed the schematic representation of CRISPR locus.

Figure 1

Schematic representation of CRISPR locus.



Note: Figure adapted from CRISPR template, by BioRender.com (2024)

The CRISPR-Cas defense mechanism can be divided into three stages including CRISPR spacer integration, CRISPR biogenesis and target interference (Hille & Charpentier, 2016; Rath et al., 2015). The first stage, also called the adaptation stage, begins with the invasion of foreign mobile genetic elements. During this stage, foreign DNA segments, known as protospacers, are identified and integrated between two neighbouring repeats in the CRISPR array by the coordinated action of Cas1 and Cas2 proteins. The protospacer adjacent motif (PAM), a conserved sequence of nucleotides located near the protospacer, serves as the recognition motif for acquiring the DNA fragment (Aydin et al., 2017). The foreign DNA is cut into short fragments by Cas proteins and integrated into the CRISPR array as new spacers, forming the basis for future immune responses. After the integration of new spacers, the second stage involves the transcription of the CRISPR array into a long precursor CRISPR RNA (pre-crRNA) by an RNA polymerase (RNAP). These pre-crRNAs are processed into smaller, functional CRISPR RNAs (crRNAs) by endoribonucleases. In the final stage, the mature crRNAs form a ribonucleoprotein complex with Cas proteins. This complex then guides the Cas proteins to the target site by recognizing and binding to complementary sequences in the invading viral or plasmid DNA. Once bound, the Cas proteins cleave the foreign genetic elements, effectively neutralizing the threat and protecting the host bacterium from future invasions (Hille & Charpentier, 2016; Rath et al., 2015; Xu & Li, 2020).

The classification of CRISPR-Cas systems is primarily based on the structural and functional characteristics of the Cas proteins present within the system. To date, several classification schemes have been proposed. In the latest update, CRISPR-Cas system was classified into two main classes, 7 types and 46 sub-types (Makarova et al., 2025). The division of CRISPR-Cas systems is often based on the composition of Cas genes, type of molecules targeted by the system, differences in the maturation of CRISPR RNAs (crRNAs), dependency on host factor and composition of effector complexes, as summarized in Table 1.

CRISPR-Cas and its role in antibiotic resistance and pathogenicity

Antibiotic resistance poses a significant global health threat, and addressing this problem has led researchers to explore the potential of the CRISPR-Cas system. Bacteria acquire resistance through multiple mechanisms, including mutations that alter antibiotic target sites, enzymatic degradation of antibiotic molecules, and active efflux of drugs from the cell (Blair et al., 2015; Darby et al., 2023; Giedraitiene et al., 2011; Reygaert, 2018). To combat this issue, the CRISPR-Cas system could serve as a robust defense by targeting and eliminating genetic elements responsible for antibiotic resistance, such as plasmids or transposons, thereby restoring the efficacy of antibiotics (Botelho et al., 2023; Louwen et al., 2014). Furthermore, mobile genetic elements (MGEs) can encode Anti-CRISPR (Acr) that neutralize CRISPR-Cas activity, allowing MGEs to evade immune targeting. Acr proteins have been identified in various genetic contexts, including prophages, plasmids, and integrative and conjugative elements (ICEs). For instance, prophages in *Pseudomonas aeruginosa* encode AcrIF1, which binds the Type I-F cascade complex and blocks DNA recognition. In *P.*

aeruginosa, lysogenic bacteriophages produce AcrIF1, which binds to the cascade complex of the Type I-F CRISPR-Cas system, blocking DNA recognition and cleavage. Other prophage-encoded inhibitors like AcrIF2 and AcrIF3 disrupt interference by targeting different stages of the defense process. Moreover, Kadkhoda et al. (2024) recently reported that the Acrs could decrease the CRISPR-Cas system's activity against phages and facilitate the acquisition of antibiotic resistance genes and virulence traits for bacteria (Kadkhoda et al., 2024).

Table 1

Classification scheme of the CRISPR-Cas system.

Types	Cas endonuclease	Target	Mechanism of action	References
Class: 1 Cas protein(s): Multiple effector				
I	Cas3	DNA	• Contain a large ribonucleoprotein complex called Cascade (CRISPR-associated complex for antiviral defense) that is responsible for target DNA recognition and binding. • When the target DNA is recognized, Cascade undergoes a conformational change and recruits a Cas3 nuclease, which cleaves the target DNA, ultimately leading to the destruction of the invading viral genetic material.	Gleditzsch et al., 2019; Hille & Charpentier, 2016; Hochstrasser et al., 2014; Schwartz et al., 2022
III	Cas10	DNA, RNA	• Use a multi-subunit complex called Csm (CRISPR-associated complex for antiviral defense) that recognizes and binds to the target DNA. • -Upon target binding, the effector complex activates a Cas10 nuclease, which cleaves both the target DNA and the CRISPR RNA. • However, the mechanism by which Type III systems provide immunity is still not entirely understood.	Jia et al., 2019; Kolesnik et al., 2021; Paraan et al., 2023
IV	Csf1	-	• Employ a multi-subunit complex called C2c (Class 2 candidate), which consists of several Cas protein. • However, the exact mechanism of Type IV systems remains an area of active research.	Pinilla-Redondo, Mayo-Muñoz, et al., 2020; Taylor et al., 2021; Zhou et al., 2021
Class: 2 Cas protein(s): Single, large, multidomain				
II	Cas9	DNA	• Utilize Cas9 to target DNA, which is guided by a dual RNA molecule consisting of crRNA (CRISPR RNA) and tracrRNA (trans-activating CRISPR RNA) that combine to form a single guide RNA (sgRNA). • Once bound to the target DNA, Cas9 creates a double-stranded break (DSB) at the precise location dictated by the sgRNA, then DSB can be repaired by the cell's own DNA repair machinery, either through non-homologous end joining (NHEJ) that often results in small insertions or deletions (indels), which can disrupt the function of the target gene or homology-directed repair (HDR) that can be harnessed to introduce specific changes or insertions into the DNA for precise gene editing.	Chylinski et al., 2014; Mir et al., 2018; Tang & Fu, 2018

Table 1 (continued)

Classification scheme of the CRISPR-Cas system.

Types	Cas endonuclease	Target	Mechanism of action	References
Class: 2 Cas protein(s): Single, large, multidomain				
V	Cas12	DNA	• Cas12 is guided by a single crRNA, which, unlike the dual RNA of Cas9, includes both the guide sequence and a partially complementary sequence that forms a hairpin structure that is critical for activating Cas12's nuclease activity. • Once Cas12 binds to the target DNA, it cleaves both strands, creating staggered ends. Similar to Cas9, these DSBs can be repaired by the cell's repair machinery.	Paul & Montoya, 2020; Tang & Fu, 2018; Tong et al., 2021; Yan et al., 2019
VI	Cas13	Single stranded RNA	• Use a single effector nuclease called Cas13, which targets RNA, guided by crRNA. Upon target RNA binding, it undergoes a conformational change that activates its RNA-cleaving activity, effectively destroying the viral RNA.	O'Connell, 2019; Perčulija et al., 2021

Numerous research studies have explored the impact of CRISPR-Cas systems on the restriction of horizontal genetic transfer (HGT) through genomic analyses (Pinilla-Redondo et al., 2020; Watson et al., 2021; Wheatley & MacLean, 2020). The genomic and phylogenetic analyses of CRISPR-Cas systems provide insights into their role in limiting HGT; however, experimental validation is essential to confirm their impact on AMR and whether spacers indirectly target regions linked to AMR genes rather than the genes themselves (Faure et al., 2019). Integrating genomic analyses with controlled laboratory experiments is therefore essential to validate CRISPR-Cas systems as tools to prevent and control AMR spread while ensuring specificity and minimizing off-target effects.

In contrast to viral-mediated immunity, which can be evaded by viral mutations, overcoming immunity against elements like plasmids often necessitates the loss or deactivation of the CRISPR-Cas system itself. Viruses, particularly bacteriophages, rapidly mutate to escape immune recognition by altering their surface proteins or target sequences, enabling quick adaptation to host defense (Marraffini, 2015). Additionally, phages can produce anti-CRISPR proteins that inhibit CRISPR-Cas systems, by parasitizing the host's translation machinery, thereby bypassing bacterial immunity and allowing continued infection (Pawluk et al., 2016).

CRISPR-Cas systems found in bacteria on the WHO list of global priority pathogens

The CRISPR-Cas system has been identified in various bacterial species, including those on the 2017 World Health Organization (World Health Organization, 2017) global priority list of antibiotic-resistant pathogens (Table 2). Generally, these pathogens are highly resistant to antimicrobials which often include last-line agents such as carbapenems, colistin and third-generation cephalosporins, resulting in limited choice of effective treatment options (World Health Organization, 2024). These pathogens are responsible for severe infections and are often associated with significant morbidity and mortality. The listed pathogens are also characterized by the capacity to acquire and spread resistance determinants through HGT, integrons and other mobile genetic elements (Partridge et al., 2018).

Several studies have shown that the presence of CRISPR-Cas systems can lead to reduced HGT, limiting the exchange of resistance genes among bacterial populations (Louwen et al., 2014; Pursey et al., 2022; Tao et al., 2022). This natural defense mechanism may help to mitigate the rapid spread of antibiotic resistance. Studies on specific bacterial species, such as *S. pyogenes* and *S. aureus*, have provided compelling evidence for the role of CRISPR-Cas in regulating antibiotic resistance gene transfer (Le Rhun et al., 2019; Pursey et al., 2022). In *S. pyogenes*, the presence of a functional CRISPR-Cas system has been linked to lower rates of plasmid acquisition, leading to reduced antibiotic resistance spread in clinical isolates. Similarly, Wheatley and MacLean reported that CRISPR-Cas systems could restrict HGT in *P. aeruginosa* while Klebsiella pneumoniae strains that possess CRISPR-Cas systems tend to show lower levels of antibiotic resistance than CRISPR-Cas–negative strains, and the presence of CRISPR-Cas has been associated with reduced acquisition of blaKPC-carrying plasmids (Wheatley & MacLean, 2020). This pattern suggests that CRISPR-Cas activity may help preserve the genomic stability of more antibiotic-sensitive lineages by limiting the uptake of resistance plasmids

(Mackow et al., 2019). In addition, a negative correlation was observed between the lack of a CRISPR-Cas system and antibiotic resistance in clinical isolates of *Enterococcus faecalis* and *Enterococcus faecium* (Palmer & Gilmore, 2010; Tao et al., 2022). This suggests that CRISPR-Cas might act as a preventive mechanism, hindering certain isolates from acquiring specific virulence factors.

Table 2

Distribution of CRISPR-Cas systems among bacteria mentioned in the WHO global priority pathogen list

WHO global priority pathogens	CRISPR type and subtype	Cas proteins	References
Critical			
Acinetobacter baumannii	I-F1, I-F2, I-b	cas1, cas3, csy1, csy2, csy3, and cas6f	Guo et al., 2022; Mortensen et al., 2021; Silva et al., 2023; Tyumentseva et al., 2021
Pseudomonas aeruginosa	I-C, I-E, I-F	cas3, cse1. Cse2, cse2, cas7, cas5, cas6e, cas1, cas2, cas6c, cas4	Luz et al., 2019; Mortensen et al., 2021; Silva et al., 2023; Silveira et al., 2020; Van Belkum et al., 2015
Enterobacteriaceae family			
Klebsiella pneumoniae	I-E, I-E*, I-F	cas1, cse1, cas3	Li et al., 2018a; Mackow et al., 2019; Wang et al., 2020b
Escherichia coli	I-E, I-F	csy1, cas1	Aydin et al., 2017
Serratia spp.	I-E, I-F, I-C	cas1, cas3	Kushwaha et al., 2022
Proteus spp.	I-E	-	Kushwaha et al., 2022
High			
Enterococcus faecium	II-A	csn 1, cas1, cas2, csn 2	Gholizadeh et al., 2020; Lindenstrauß et al., 2011; Mortensen et al., 2021; Palmer & Gilmore, 2010
Staphylococcus aureus	II-C, III-A	cas1, cas2, cas10, cas11, cas7, cas5, cas7, csm6, cas6	Li et al., 2021; Mortensen et al., 2021
Helicobacter pylori	-	-	-
Salmonella spp.	I-E	cas3, cas83, cse2gr11, cas7, cas5, cas6e, cas1, cas2	Tanmoy et al., 2020
Neisseria gonorrhoeae	-	-	-
Medium			
Streptococcus pneumoniae	absence	absence	Lemaire et al., 2022
Haemophilus influenzae	-	-	-
Shigella spp.	I-E	cas3, cse1, cse2, cas7, cas5, cas6e, cas1, cas2, cas7,	Guo et al., 2015

Note: I-E indicates a variant subtype of the Type I-E CRISPR-Cas system that differs from the canonical Type I-E system in its cas gene organization and genomic localization.*

Therefore, understanding how CRISPR-Cas systems function and their association with antibiotic resistance in different bacterial species may provide innovative strategies for combating bacterial infections and addressing antibiotic resistance. Moreover, the CRISPR-Cas system's potential applications in gene editing and gene therapy could pave the way for future ground-breaking medical treatments.

In addition to its role in antibiotic resistance, the CRISPR-Cas system influences bacterial pathogenicity in several ways. For instance, they can regulate the expression of virulence genes, which are responsible for the ability of bacteria to cause disease. In some cases, CRISPR-Cas-mediated interference with specific virulence genes can attenuate the pathogenicity of the bacterium, making it less harmful to the host. This include down regulating genes in *E. coli* that are involved in membrane integrity and adherence to host cells (Rodrigues et al., 2023) causing bactericidal effects in a mouse skin colonization model by targeting *S.aureus* virulence genes (Bikard et al., 2014). Conversely, in other instances, the CRISPR-Cas system may enhance virulence; a *E.faecalis* strain harboring CRISPR-Cas was better able to colonize a host by forming biofilms compared to strains lacking the system (Wu et al., 2022).

On the other hand, the CRISPR-Cas system can also impact the host immune response during bacterial infection. By targeting and cleaving host immune-related genes or regulatory elements, the CRISPR-Cas system can suppress the host immune response, allowing the bacterium to evade the immune system and establish a successful infection. As such, the repression of the production of bacterial lipoprotein controlled by CRISPR-Cas9 system allowed *Francisella novicida* to evade the host's immune response. The study marked the first demonstration of Cas9 regulating a bacterial gene (Marraffini, 2013). In addition, deletion of Cas9 in *Neisseria meningitidis* affected the virulence traits such as adherence to and invasion of human epithelial cells in the pathogenesis of invasive meningococcal disease (Heidrich et al., 2019).

The CRISPR-Cas system can be used to re-sensitize drug-resistant bacteria to antibiotics by site-specific cleavage of crucial domains of their genome or specifically disabling plasmids carrying antibiotic resistance genes. Furthermore, CRISPR-Cas systems are in constant evolutionary arms races with bacteriophages and plasmids. Spacer acquisition is an active process that involves the concerted activity of the Cas1/Cas2 complex to integrate foreign DNA into the array. As these genetic elements evolve, they may develop mechanisms to evade CRISPR-Cas targeting. This interplay can influence the virulence of the bacterium by affecting the ability of phages and plasmids to transfer genes related to pathogenicity. On the positive side, CRISPR-Cas systems have been harnessed as a tool for detecting and diagnosing pathogenic bacteria. Researchers have developed CRISPR-based diagnostic tools, such as SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing), which utilize CRISPR-Cas systems to detect specific genetic sequences unique to pathogenic bacteria (Huang et al., 2022; Vangah et al., 2020; Mustafa & Makhawi, 2021). This approach enables rapid and accurate identification of bacterial pathogens, facilitating early intervention and treatment (Pardee et al., 2016; Wang & Li, 2021).

Detection of pathogens

Nucleic acid amplification is one of the most valuable tools across all fields of biomedical sciences ranging from biotechnology to diagnosis of infectious diseases (Lee et al., 2021). Several amplification methods have been reported including polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA) and strand displacement amplification (SDA). Among these methods, PCR is the most widely used in various forms such as nested PCR (nPCR), multiplex PCR (mPCR), and reverse transcription PCR (RT-PCR) (Lau et al., 2020). During the recent coronavirus 2019 (Covid-19) outbreak, PCR-based detection methods were regarded as the gold standard for detection of SARS-CoV-2. In fact, PCR-based methods have been widely used in all fields of science to amplify a target sequence. Although these methods offer many advantages, they still suffer from insufficiencies and inadequacies due to the requirement of a sophisticated instrument for amplification thus limiting its application in resource limited settings (Puig-Serra et al., 2022). Hence, it is crucial to search for an alternative method that is user-friendly, rapid, cost-efficient, has high sensitivity and can be performed without the need of a sophisticated instrument or power supply. In recent years,

CRISPR-Cas systems such as Cas9, Cas12, Cas13, and Cas 12f have emerged as precise and adaptable molecular tools. As newly emerging multifunctional toolboxes, CRISPR-Cas systems have been harnessed beyond their traditional applications of gene editing (Wang et al., 2020a), enabling a wide range of novel applications such as epigenome modification, disease modelling, bioimaging, and nucleic acid detection (Li-Juan et al., 2017; Makarova et al., 2019). Among these applications, CRISPR-Cas system has been widely utilized for nucleic acid detection particularly to identify and categorize antimicrobial resistant pathogens (Ali Agha et al., 2025). Among the diverse CRISPR systems, those belonging to class 2 are the most widely used for nucleic acid detection and applied for diagnostic purposes, as these systems are simpler to reconstitute (Kaminski et al., 2021). Each of these CRISPR-Cas diagnostic tools had been carefully designed to leverage specific Cas effector characteristics (Cas9, Cas12a, Cas13 and Cas12f) and optimized in terms of sensitivity, specificity, turnaround time, and ease of use. Together, they represent a significant advancement and hold promise as a new alternative for clinical applications (Ali Agha et al., 2025).

CRISPR-Cas9-based diagnostic methods

The first developed CRISPR-based diagnostic methods mainly used the Cas9 enzyme, a type II protein. The Cas9 protein recognizes a PAM on the target DNA. Once bound, the Cas9 enzyme uses two domains, namely histidine–asparagine–histidine (HNH) that cleaves the DNA strand (target strand) complementary to the single guide RNA (sgRNA) sequence specific for type II (see Table 1) and a RuvC nuclease domain which is required for cleaving the non-complementary strand (non-target strand), resulting in double-stranded DNA breaks (Figure 2a). This break is then repaired by the cell's natural DNA repair machinery (Jinek et al., 2014). Thus, the Cas9 enzyme is widely adopted for specific cleavage and DNA binding-based diagnostics. The combination of CRISPR-Cas9 with nucleic acid amplification methods such as PCR and loop-mediated thermal amplification (LAMP) is commonly used for the detection of specific DNA and RNA sequences. Cas9 is characterized by its high sequence specificity and ability to differentiate single-base mismatches (Anderson et al., 2015). In 2016, Pardee et al. introduced a CRISPR-Cas9 based method dubbed as nucleic acid-sequence based amplification CRISPR cleavage (NASBACC) for detection and discrimination of American and African strains of the Zika virus. This method incorporates the specific cleavage ability of Cas9 and a toehold switch sensor on a freeze-dried paper-based platform which allows rapid and robust detection of RNA targets with high sensitivity and specificity (Pardee et al., 2016). In brief, extracted RNAs targeting the binding of the RNA ribo-regulators were isothermally amplified, via NASBA. A trigger sequence of the toehold switch sensor was added after NASBA amplification. Next, sgRNA was designed with a strain-specific PAM sequence targeting specifically American Zika virus strains. With the presence of the designed sgRNA, Cas9 was only able to cleave the amplified products of American Zika virus strains and not the African strains. Concurrently, the toehold switch sensor for sequence-based detection of Zika virus was designed. Toehold switch sensors are synthetic RNAs that bind to target mRNAs. They contain a hairpin structure containing a ribosome-binding site (RBS) and start codon, followed by the sequence of a reporter, in this case LacZ. The reporter is only translated upon addition of the trigger sequence, which by binding to the sensor stretches the hairpin structure thus making the RBS and start codon available. Translation of LacZ results in a colorimetric output on a test paper through the conversion of a yellow substrate to purple products. The discrimination of different viral strains is crucial for genotyping and identifying the origin of an infection. In addition, NASBACC eliminates the requirement for a thermal cycler as it runs under isothermal conditions, making it suitable for point-of-care applications (Pardee et al., 2016; Song et al., 2021). Figure 3a showed the schematic representative diagram of NASBACC mechanism.

Following the development of NASBACC, CRISPR-Cas9 combined with various other types of isothermal amplification technologies such as exponential amplification reaction (EXPAR), rolling circle amplification (RCA) and strand displacement amplification (SDA) were also introduced. These methods were reported to possess high sensitivity and specificity as nucleic acid detectors (Chakraborty et al., 2022). In 2018, Huang and colleagues introduced a method known as CRISPR-EXPAR for site-specific nucleic acid detection and demonstrated its power for the detection of DNA methylation and *Listeria monocytogenes* mRNA. CRISPR-EXPAR is a single-stranded nucleic acid biosensing method that combines the advantages of CRISPR-Cas9 site-specific cleavage and rapid exponential amplification kinetics (EXPAR). Briefly, the Cas9/sgRNA complex performs site-specific cleavage of single-stranded DNA (ssDNA) substrates, generating cleaved fragments referred to as X. These fragments then hybridize with the EXPAR template and are extended from their 3' ends along the template by DNA polymerase. The resulting duplex is subsequently nicked by a nicking endonuclease (NEase), releasing a copy of the fragment X from the template through the action of Vent (exo) DNA polymerase. The dissociated X then initiates a new amplification circuit (Huang et al., 2018). Due to the programmable cleavage mode of Cas9/sgRNA, it is noteworthy that the CRISPR-EXPAR could detect any site of the target sequences. In recent years, CRISPR-EXPAR has also been utilized as a tool to detect mutations in DNA of organisms.

Since the discovery of *S.pyogenes* Cas in 2012, the remarkable and customizable precision of CRISPR system has sparked innovative applications of their enzymes beyond genome engineering (Jinek et al., 2012). In 2019, Quan et al., introduced FLASH (Finding Low Abundance Sequences by Hybridization) as a next generation of CRISPR-Cas diagnostic method to enrich low abundance AMR pathogen sequences. This method can rapidly identify any drug-resistant microbes that are present in body fluids or other clinical samples, even when the bacterial or viral loads of these pathogens are so little that they elude the standard detection methods. The FLASH method uses recombinant Cas9 enzyme along with multiplex guide RNA to eliminate the background sequences for precise identification of a pathogen (Quan et al., 2019). In brief, the process begins with the treatment of genomic DNA or complementary DNA (cDNA) using phosphatase enzymes, which serve to block the DNA by removing phosphate groups from the 5' ends. This step prevents unwanted ligation and prepares the DNA for subsequent steps. Next, the treated DNA is digested using the Cas9 enzyme, which is guided by a set of specific sgRNAs designed to target particular genes of interest within the genome. The Cas9/sgRNA complex binds to the target sequences and introduces double-stranded breaks at these specific sites. Following digestion, sequencing adaptors are ligated to the ends of the cleaved DNA fragments. These adaptors are necessary for the next stages of the process, as they enable the DNA fragments to be compatible with sequencing platforms. The adaptor-ligated DNA fragments are then subjected to PCR amplification. This amplification step increases the quantity of the target DNA fragments, making them easier to detect and sequence.

Finally, the amplified DNA fragments are sequenced using high-throughput sequencing technologies (Quan et al.,

2019). This method demonstrated its ability to go beyond other CRISPR-method based diagnostic tools as it allows high levels of multiplexing with reinforcement of accuracy and sequence identity confirmation that is intrinsic to a traditional next-generation sequence readout. This method has been adapted for direct testing of clinical cases including patients with vancomycin-resistant E. faecium and MRSA infections (Quan et al., 2019). In the same year, another method known as CRISPR-Chip was introduced. The CRISPR-Chip couples Cas9 to a graphene-based field-effect transistor (FET) to detect *mecA* in *Staphylococcus epidermidis* electrically (Hajian et al., 2019). The technique bypasses amplification, offering label-free and real-time biosensing ideal for point-of-care use. In addition, another study also reported a related amplification-free approach using fluorescence-based Cas9 assay for *Pseudomonas aeruginosa*. The study was designed to identify carbapenemase genes such as *blaOXA*, *blaVIM*, and *blaIMP* directly from clinical samples (Fu et al., 2022). This method showcases rapid turnaround and high sensitivity, strengthening its role in PCR-independent diagnostics. CRISPR-EXPAR has since emerged as another powerful CRISPR-assisted amplification strategy. Song et al. (2021) introduced CRISPR-EXPAR, whereby the study demonstrated the application of CRISPR-EXPAR for identification of a model target mutation within the human epidermal growth factor receptor 2 (HER2) gene down to 437 aM with a reported high specificity. The study suggested that through proper design of sgRNAs, various mutations can be detected using the CRISPR-EXPAR method (Song et al., 2021). Therefore, CRISPR-EXPAR can serve as a versatile nucleic acid detection strategy for molecular diagnostic applications. The EXPAR methods leverage Cas9 to identify specific pathogen sequences in clinical samples with remarkable sensitivity (Xu et al., 2023). These studies demonstrated CRISPR-EXPAR as a versatile nucleic acid detection platform with broad potential for molecular diagnostic applications.

CRISPR-Cas12-based diagnostic methods

CRISPR-Cas12a or previously known as *Cpf1* is a class V CRISPR-Cas protein. The trans-cleavage activities of Cas12a were first discovered during the characterization of its cleavage behaviors against target ssDNA (Chen et al., 2017). Cas12a utilizes a guide CRISPR RNA (crRNA) to recognize and bind to complementary DNA sequences flanked by a PAM. Upon binding to its target DNA, Cas12a undergoes a conformational change that activates its collateral cleavage activity, leading to the non-specific degradation of nearby ssDNA molecules (Figure 2b), thus preventing false annealing in the nucleic acid detection method making it useful for nucleic acid amplifications (Mao et al., 2022). Although Cas9-based detection remains widely used, Cas12a has gained increasing adoption due to its unique features and promising outcome for nucleic acid detection of bacteria and viruses. In 2018, Chen et al. established a method known as DNA endonuclease targeted CRISPR trans-reporter (DETECTR) that involves the recognition and cleavage of target DNA or RNA sequences, followed by a reporter-based signal generation to indicate the presence of the targeted pathogens. They showed that activated Cas12a exhibits robust collateral ssDNA cleavage, a feature central to DETECTR's signal-generation mechanism. This characteristic has been effectively utilized in DETECTR, which combines Cas12a activation with isothermal amplification to provide a rapid and highly sensitive method for DNA detection. DETECTR has been successfully employed for the detection of human papillomavirus (HPV), including the differentiation between HPV16 and HPV18 strains, showcasing its potential in clinical diagnostics and pathogen detection (Chen et al., 2018). Besides HPV detection, DETECTR has also been applied for detection of African swine fever virus (ASFV), SARS-CoV-2 and *Bacillus anthracis* in studies which demonstrated high sensitivity and specificity of the DETECTR method (Broughton et al., 2020; Tsou et al., 2019; Xu & Li, 2020). Notably, DETECTR analysis only requires an hour to obtain results, thus providing a rapid detection platform for nucleic acid amplification. In addition, the DETECTR has also been utilized to detect *mecA* in *Staphylococcus aureus*, employing isothermal amplification and a fluorescent reporter for efficient and cost-sensitive output (Suea-Ngam et al., 2021). Similarly, paper-based lateral flow assays for *Neisseria gonorrhoeae* and *Salmonella enterica* utilize Cas12a to target 23S rRNA and *qnrS, bla, CTX-M*, respectively. These assays produce rapid, visible results without requiring specialized instrumentation (Cai et al., 2021; Shin et al., 2021). Figure 3b showed the schematic representative diagram of DETECTR mechanism.

HOLMES (one-HOur Low-cost Multipurpose highly Efficient System) as a nucleic acid detection method was patented (Wang et al., 2018). HOLMES applies the PCR or RT-PCR method to amplify the targeted nucleic acids and detects the amplicons with Cas12a trans-cleavage activities combined with a fluorescent reporter system. The HOLMES system employs a simple and efficient workflow that avoids extensive sample processing (Kumaran et al., 2023). Briefly, biological samples such as blood or saliva are collected and processed to extract DNA containing the target sequence of the selected bacteria. The isolated DNA is then added to a reaction mixture containing Cas12a-crRNA complexes, where the crRNA is designed to specifically bind to the target genetic region. Upon encountering the complementary target DNA, the Cas12a-crRNA complex becomes activated, triggering a conformational change. This activation initiates the nonspecific cleavage of nearby ssDNA, including a reporter molecule present in the reaction. The reporter molecule typically consists of an ssDNA with an attached fluorescent or colorimetric reporter group. As a result of the cleavage, the reporter molecule is fragmented, leading to the release or separation of the reporter group. This separation generates a detectable signal such as fluorescence or a change in color which indicates the presence of the target DNA sequence in the sample. HOLMES has been applied to detect human single nucleotide polymorphisms (SNPs) and DNA/RNA viruses as well as to differentiate between the different strains of the viruses (Shiyuan et al., 2018). In 2019, an improved version of HOLMES known as HOLMESv2 was reported by Li et al. utilizes an Cas12b effector to exclusively

identify SNPs and rapid detection of virus RNA, human cell mRNA, and circular RNA as well as precisely measuring the degree of target DNA methylation through a combination of Cas12b detection and bisulfite treatment. Remarkably, the HOLMESv2 detection process was simplified by substituting PCR amplification with LAMP-based amplification. To create a more streamlined one-pot detection system, the researchers combined LAMP, which operates at constant temperature, with a stable Cas12 effector called AacCas12b from *Alicyclobacillus acidoterrestris*. Unlike traditional RNA detection methods that require a reverse transcriptase for generating cDNA templates for LAMP amplification, HOLMESv2 utilized *Bst* 3.0 DNA polymerase capable of recognizing both RNA and DNA templates, thus eliminating the need for a separate reverse transcription step (Li et al., 2019). Expanding versatility, Cas12a has been incorporated into smartphone-compatible platforms such as the system targeting *tet(O)* in *Campylobacter jejuni*, enabling highly portable, real-time detection for on-site surveillance and field applications (Li et al., 2022). Figure 3c showed the schematic representative diagram of HOLMES mechanism.

CRISPR-Cas13-based diagnostic methods

Cas13 comprising Cas13a, Cas13b, Cas13c, and Cas13d is a type VI RNA-guided RNase with the ability to cleave single-stranded RNA (ssRNA). The Cas13 consists of two higher eukaryote and prokaryote nucleotide-binding (HEPN) domains which is commonly found in the ssRNA specific endoribonucleases. Cas13 primarily targets RNA and initiates binding upon recognizing a protospacer flanking site (PFS) on the target RNA molecule. Upon binding, Cas13 undergoes a conformational change that activates its collateral cleavage activity. This collateral cleavage capability enables Cas13 to cleave nearby ssRNA molecules in a non-specific manner (Figure 2c). This property has significant utility and can be harnessed for detecting specific RNA sequences by linking it to a ssRNA reporter molecule. This reporter molecule serves as a detection mechanism, as its cleavage by activated Cas13 generates a detectable signal, facilitating the identification of the target RNA sequences in diagnostic assays or research applications (Kwon & Shin, 2021). In 2016, East-Seletsky et al., utilized Cas13a from *Leptotrichia buccalis* (LbuCas13a), an RNA-guided RNA endonuclease, to detect phage λRNA and endogenous β-actin mRNA. This enzyme exhibits a specific cleavage ability on ssRNA guided by crRNA. Interestingly, the study indicated that even after its initial cleavage activity is completed, Cas13 is still able to maintain the non-specific endonuclease activity, thus enabling it to continue cleaving other non-target ssRNAs. The detection method relies on the activation of Cas13a's trans-cleavage activity by the target ssRNA, leading to the degradation of fluorescence-quenched-group-labelled ssRNA. This process releases a fluorescence signal, allowing for rapid and sensitive detection (East-Seletsky et al., 2016). The identification of this collateral activity, linking accurate target recognition to a more extensive RNase activity capable of cleaving reporter molecules, has paved the way for a ground-breaking method in nucleic acid detection.

The first Cas13a-based nucleic acid detection platform, termed as the Specific High-sensitivity Enzymatic Reporter unLOCKing (SHERLOCK) platform, was introduced in 2017 by Gootenberg and colleagues (Gootenberg et al., 2017). SHERLOCK applies the same principle as DETECTR whereby both methods utilize a fluorescence reporter system with recombinase polymerase amplification (RPA)-based target amplification. The main difference between SHERLOCK and DETECTR lies in the fact that SHERLOCK requires additional steps of in vitro transcription using T7 RNA polymerase to convert amplified DNA to RNA (Kwon & Shin, 2021). To date, SHERLOCK has been applied for detection of dengue virus (DENV) and zika virus (ZIKV) (Gootenberg et al., 2017; Kellner et al., 2019; Myhrvold et al., 2018). In addition, the SHERLOCK lateral flow system was also used to detect *blaCTX-M* in foodborne pathogens like *E. coli* and *Salmonella*. It combines duplex PCR with lateral flow strips demonstrating high sensitivity, making it ideal for field surveillance and food safety monitoring (Hatrongjit et al., 2025).

Interestingly, researchers have introduced a pathogen-sample treatment method known as HUDSON (Heating Unextracted Diagnostic Samples to Obliterate Nucleases), which allows sample to be detected from body fluids without nucleic acid extraction (Freije et al., 2019). The lyophilization of SHERLOCK reagents also enables them to be preserved for extended periods of time, making them well-suited for long-term storage. Besides, these reagents can be easily applied to paper strips, facilitating their use in field applications. However, despite its success in targeting RNA for diagnostic purposes, SHERLOCK has several limitations such as the lack of quantitation and reliance on fluorescent detection for read-out (Kwon & Shin, 2021). To overcome the limitations of the existing method, updated versions of SHERLOCK i.e. SHERLOCKv2 were developed. In SHERLOCKv2, a combination of multiple fluorescence reporters such as Cy5, FAM, HEX and TEX allow simultaneous detection of ZIKV and DENV. By reducing the primer concentration and input sample amount during target pre-amplification, a higher sensitivity was obtained. Primarily, Cas13 recognizes and binds to specific RNA, activating its collateral cleavage activity to cut nearby RNA molecules. These RNA fragments then activate Csm6, an auxiliary CRISPR-associated enzyme which further cleaves additional RNA, amplifying the detection signal. The incorporation of both enzymatic activity of Cas13 and Csm6 significantly enhanced the sensitivity of detection by more than 3.5 times. This enables the detection of extremely low target RNA concentrations, down to the attomolar range (Gootenberg et al., 2018). The utilization of lateral flow paper strips enabled SHERLOCKv2 to rapidly detect ZIKV and DENV within 90 minutes, with a remarkable sensitivity of 2 aM without the need for additional equipment (Myhrvold et al., 2018). The updated version of SHERLOCKv2 addresses the limitation of SHERLOCK by eliminating the need for fluorescence detection equipment as well as performing both quantitative and large-scale

detection of variety of pathogens. It is noteworthy that SHERLOCKv2 technology is the first CRISPR gene editing technology that received authorization from the US-FDA for emergency use as a SARS-CoV2 detection kit (Mahas et al., 2021).

In 2023, the study by Liang et al. presented a CRISPR-Cas13a fluorescence assay designed for the rapid and accurate detection of the *blaKPC* gene in carbapenem-resistant *Klebsiella pneumoniae*. The method integrates recombinase polymerase amplification (RPA) with Cas13a-mediated collateral cleavage, which activates a fluorescent reporter upon recognition of the target RNA. The assay was tested on 311 sputum samples, confirming its robustness across diverse clinical matrices. The entire workflow is reported to take around one hour (Liang et al., 2023), making it a powerful tool for hospital-based surveillance and a timely decision-making therapeutic in managing carbapenem-resistant infections.

CRISPR-Cas 12f-based diagnostic methods

CRISPR-Cas12f or formerly known as Cas14, comprises 24 variants clustered into three subgroups: Cas12f1 (previously Cas14a), Cas12f2 (Cas14b), and Cas12f3 (Cas14c), all of which share a conserved RuvC nuclease domain (Harrington et al., 2018; Karvelis et al., 2020). Initially discovered in the DPANN superphylum—a group of extremophile archaea—Cas12f is notably smaller (40–70 kDa, 400–700 amino acids) than other CRISPR-associated proteins, earning it the designation of a miniature CRISPR system (Harrington et al., 2018). Unlike Cas9 and Cas12a, Cas12f uniquely targets single-stranded DNA (ssDNA) without requiring a protospacer adjacent motif (PAM), significantly expanding its targeting flexibility (Takeda et al., 2021). Despite its small size, Cas12f exhibits higher specificity in cleavage compared to Cas9 and Cas12, making it advantageous for precision genome editing and diagnostics (Wu et al., 2023). Cas12f relies on tracrRNA and crRNA to recognize and cleave ssDNA (Figure 2d). This capability is particularly valuable for targeting ssDNA viruses (e.g., parvoviruses) and mobile genetic elements (e.g., plasmids) that replicate via ssDNA intermediates (Harrington et al., 2018; Zhang et al., 2021) . Its PAM-independent activity broadens its utility in genetic engineering, especially in AT-rich genomic regions where PAM sequences are scarce (Kim et al., 2022).

The first diagnostic application of Cas12f was demonstrated by Harrington et al. (2018) in combination with DETECTR, detecting SNPs in the HERC2 gene linked to blue-eyed traits. Subsequent studies have explored its use in pathogen detection, including DNA viruses (e.g., HPV, hepatitis B virus) (Chen et al., 2021; Li et al., 2018b). Unlike traditional PCR, Cas12f-based diagnostics offer rapid, equipment-free detection, making them suitable for point-of-care testing (POCT) in low-resource settings (Chen et al., 2023). Recent advancements have highlighted the potential of Cas12f in antimicrobial resistance (AMR) surveillance. Notably, a CRISPR-Cas14a-based platform was developed for the direct detection of rifampicin (RIF) and isoniazid (INH) resistance mutations in *Mycobacterium tuberculosis* (Mtb) from sputum samples, demonstrating high sensitivity and specificity without the need for DNA extraction or amplification (Xiao et al., 2025). Additionally, an innovative delivery system combining CRISPR-Cas12f was designed to combat AMR in Gram-negative bacteria, showcasing the versatility and potential of Cas12f in addressing antibiotic resistance challenges (Long et al., 2024).

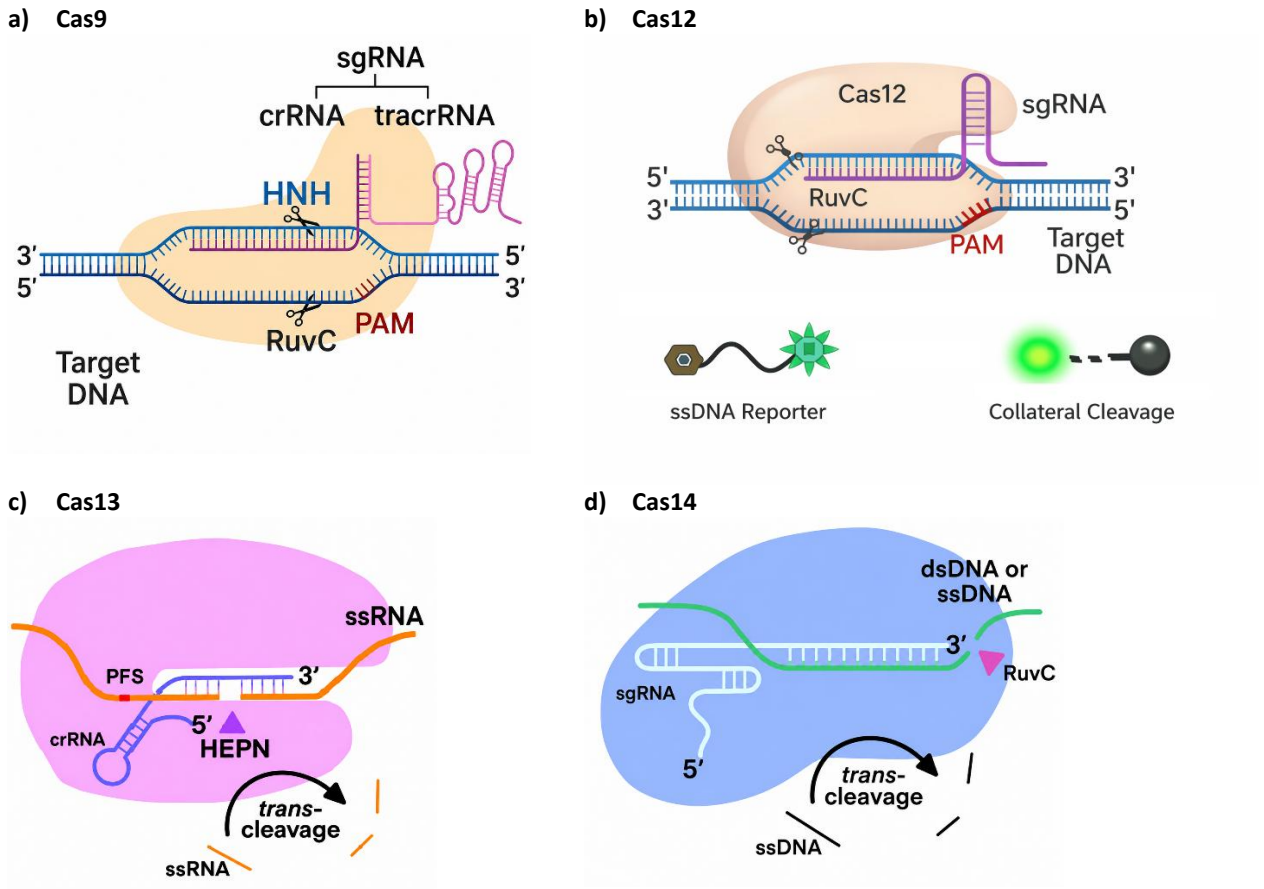
Challenges and future perspective of the CRISPR-Cas detection system toward next-generation diagnostic

The CRISPR-Cas detection system holds tremendous promise for next-generation diagnostics as it has demonstrated numerous favourable attributes such as cost-effectiveness, simplicity of operation and high sensitivity and specificity (Aman et al., 2020). The nucleic acid detection based on CRISPR-Cas systems is summarized in Table 3. However, it also faces several challenges that need to be addressed for its optimal integration into healthcare. Generally, research on the CRISPR-Cas detection system is still considered relatively new and primarily confined to laboratory experimentation. One of the primary challenges is the potential for off-target effects, where the CRISPR system may unintentionally modify genomic sequences similar to the target, leading to inaccuracies in diagnostic results. Researchers are actively working on refining CRISPR technologies to improve target specificity, incorporating modified Cas proteins and advanced computational algorithms to minimize off-target effects. Delivery efficiency poses another significant challenge, as the precise introduction of CRISPR components into target cells or tissues is crucial for the system's effectiveness. Ongoing research explores innovative delivery methods in order to optimize the process and enhance the overall efficiency of CRISPR-based diagnostics (Rasul et al., 2022). Secondly, Cas is specifically designed for nucleic acid detection and cannot be used for detecting non-nucleic acid targets. To enable the detection of non-nucleic acid targets, researchers need to design nucleic acid aptamers that can indirectly detect the target. However, the introduction of PAM has the potential to modify the aptamer's functionality, leading to a decrease in its binding ability to the target (Tan et al., 2015). The scalability of CRISPR-based diagnostics is also a concern, particularly as the demand for high-throughput screening and simultaneous detection of multiple targets grows. Innovations are needed to handle larger sample volumes and diverse target sequences without compromising the speed and accuracy of diagnostics. Additionally, achieving standardization and enabling multiple and quantitative detections also remains a challenging task. The CRISPR-Cas system is mostly established in combination with PCR amplification, RPA or other amplification methods; henceforth, these methods usually suffer from issues such as secondary structures of primers or templates and contaminants, which undoubtedly increase the complexity of detection (Wang et al., 2023). Apart from this, ethical

considerations, including issues related to privacy, consent, and the potential misuse of gene-editing technologies, present challenges to the widespread adoption of CRISPR-based diagnostics. Developing robust ethical frameworks and regulatory guidelines is crucial to navigating these complex issues and ensuring responsible use (Rasul et al., 2022).

Figure 2

Schematic representation of different types of CRISPR-based mechanism systems.



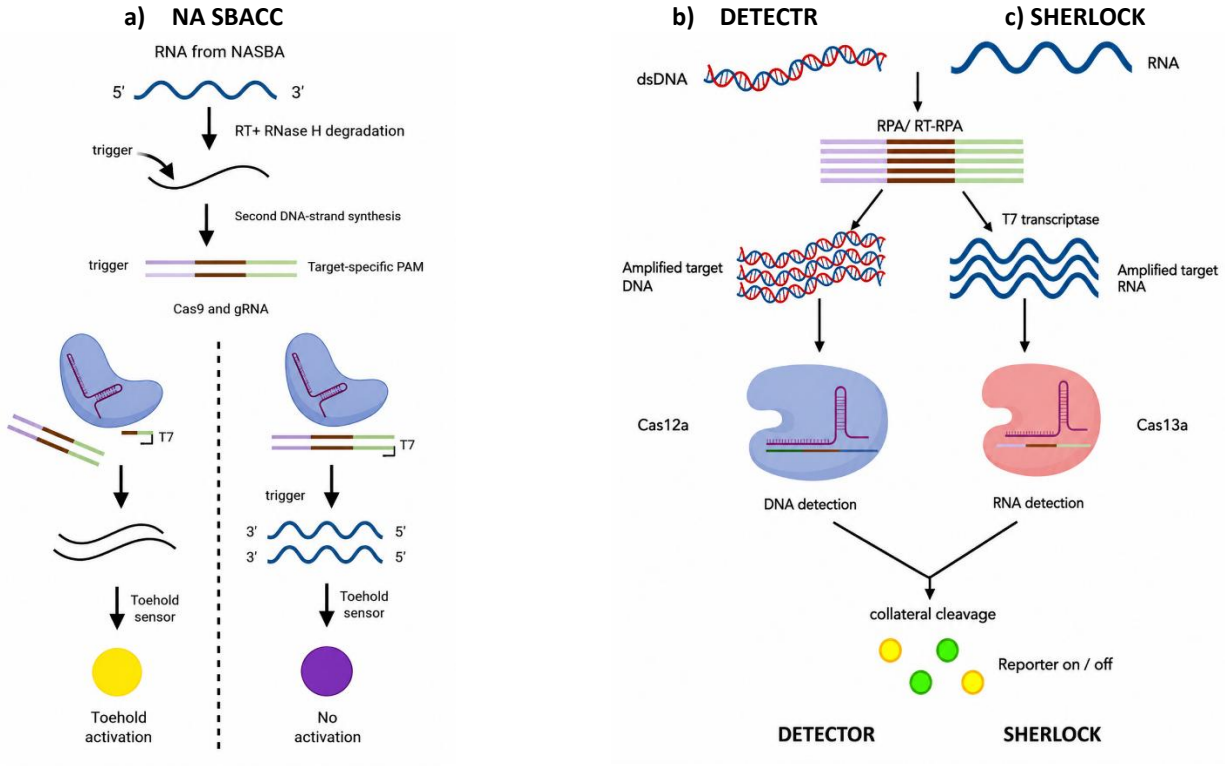
Note: a) Cas9: Involves the formation of a Cas9-sgRNA complex, which binds to the target DNA sequence with the help of PAM recognition. Once bound, Cas9 cleaves the target DNA at a specific site, guided by complementary base pairing between the sgRNA and the target DNA sequence. The HNH and RuvC nuclease domains within Cas9 are responsible for cleaving the target and non-target DNA strands, respectively, resulting in a double strand break at the target site. b) Cas12: Involves the formation of a Cas12-sgRNA complex, which binds to the target DNA sequence with the help of PAM recognition. Once bound, Cas12 cleaves the target DNA at a specific site, guided by complementary base pairing between the sgRNA and the target DNA sequence. The collateral cleavage activity of Cas12 leads to cleavage of nearby ssDNA molecules, including the ssDNA reporter, generating a detectable signal for nucleic acid detection. c) Cas13: Involves the formation of a Cas13-crRNA complex, which binds to the target RNA sequence with the help of PFS recognition. Once bound, Cas13 employs HEPN to cleave the target RNA at a specific site, guided by complementary base pairing between the crRNA and the target RNA sequence. This targeted cleavage ensures precise RNA interference. Furthermore, activated Cas13 exhibits collateral cleavage activity, leading to non-specific cleavage of nearby single-stranded RNA (ssRNA) molecules. This trans-cleavage capability expands the utility of the CRISPR-Cas13 system as a versatile tool for organism detection applications. d) Involves the formation of a Cas14-sgRNA complex, which binds to the target DNA sequence guided by complementary base pairing. Once bound, Cas14 cleaves the target DNA at specific sites, facilitated by its RuvC-like nuclease domain. The collateral cleavage activity of activated Cas14 leads to non-specific cleavage of nearby ssDNA molecules. This collateral cleavage activity broadens the utility of the CRISPR-Cas14 system, allowing for both targeted and non-specific DNA cleavage. Figure adapted from CRISPR template (BioRender.com, 2024).

Nevertheless, looking towards the future, the perspective for CRISPR-Cas detection systems is bright and optimistic. The CRISPR-based detection methods are poised to redefine the landscape of molecular diagnostics, offering unprecedented accuracy, speed, and scalability. One of the most promising applications lies in the early detection of infectious disease agents and antimicrobial resistance. Researchers are actively exploring strategies to enhance

sensitivity, specificity, and scalability of the detection methods, coupled to innovations in optimizing the diagnostic workflow as technologies evolve. In addition, the integration of artificial intelligence and machine learning is anticipated to further optimize data analysis and improve the accuracy of diagnostic results. As advancements continue, CRISPR-based diagnostics could revolutionize personalized medicine and be expanded to environmental monitoring, including agriculture fields, offering precise and accessible solutions to a myriad of challenges. While we recognize the exceptional capabilities demonstrated by the current CRISPR-Cas systems, it remains uncertain if they genuinely embody a ground-breaking, advanced technology for diagnostics until they prove efficient across various intricate domains or environments. We can only consider these tools as revolutionary next-generation diagnostics once they successfully detect a broader range of diverse targets with enhanced precision and sensitivity, enabling their transition from laboratory settings to practical or clinical applications. The reported CRISPR-based assays, such as DETECTR and SHERLOCK, harness the programmable nature of CRISPR-Cas systems to target specific DNA or RNA sequences, enabling the identification of disease-related genetic markers and antimicrobial resistance with remarkable precision.

Figure 3

Schematic representative diagram of the known different CRISPR-based diagnostic platforms for nucleic acid detection.



Note: a) NASBACC: The process of NASBA involves the amplification of RNA targets. Reverse transcription (RT) converts RNA into complementary DNA (cDNA) using a primer with a specific target sequence and an attached trigger sequence crucial for the toehold sensor. RNase H degrades the RNA in the RNA/DNA hybrid, allowing a T7 promoter-containing primer to bind and generate a complementary DNA strand. T7 transcription utilizes the double-stranded DNA (dsDNA) template to produce the target RNA sequence. The presence of a PAM sequence (shown in blue) within the dsDNA amplicon can lead to Cas9-mediated cleavage, resulting in truncated RNA lacking the trigger sequence, preventing toehold sensor activation. In the absence of the PAM sequence, full-length target RNA is transcribed during T7 transcription, activating the toehold sensor, leading to a visible colour change. b) DETECTR: The Cas12a-gRNA complex serves as a molecular sentinel that identifies specific target DNA sequences amplified by RPA. The role of RPA is to produce sufficient quantity of the target DNA to be detected. When the target DNA is detected and amplified, Cas12a becomes activated, triggering its collateral cleavage activity. This enzymatic activity results in the cleavage of nearby non-targeted single-stranded DNA molecules. Such cleavage leads to the release of detectable signals, commonly fluorescence reporters, providing a visible indication of the presence of the target sequence. ***c)*** *SHERLOCK: The Cas13a-gRNA complex functions as a molecular recognizer, binding to specific target RNA sequences transcribed from DNA amplified by RPA or RT-RPA. Upon target RNA binding, Cas13a undergoes activation, initiating collateral cleavage of nearby RNA molecules. This collateral cleavage activity results in the release of fluorescence reporters, serving as a visible indicator of the target sequence's presence. SHERLOCK thus leverages this enzymatic activity to provide sensitive and specific detection of nucleic acid targets. Figure adapted from CRISPR template (BioRender.com, 2024).*

Table 3

Summary of nucleic acid detection systems based on CRISPR-Cas.

System name	Effector	Detection method	Target detection	Advantages	Limitations	References
NASBACC	Cas9	Colorimetric detection	Zika virus, MRSA	Programmable portable sensor, direct DNA targeting, high specificity	Limited detection sensitivity for low-abundance targets	Pardee et al., 2016
CRISPR-Chip	Cas9	Electrical readout	*Staphlococcus epidermidis (mecA)*	High specificity, minimal sample prep	Requires graphene-based field, limited multiplex capability	Hajian et al., 2019
FLASH	Cas9	High-throughput sequencing (sequence readout)	MRSA, VRE	High sensitivity, flexibility without optimization, high level of multiplexing	Requires primer optimization	Quan et al., 2019
EXPAR	Cas12a	Real-time fluorescence monitoring	DNA methylation, L. monocytogenes total RNA	High sensitivity for RNA detection, rapid detection, simplicity, POC use	Limited to RNA detection, limited multiplexing capability	Song et al., 2021
Cas 12a-based portable smartphone detection	Cas 12a	Mobile interface integration	*Campylobacter jejuni (tetO)*	Ultra-portable, accessible, cost-effective	Requires app calibration	Li et al., 2022
DETECTR	Cas12a	Fluorescence-based detection	HPV, SARS-CoV-2, *Bacillus anthracis* *Staphylococus aureus (mecA)*	High sensitivity and specificity, portable, user-friendly, POC use Cost-effective, precise, suitable for quantification	Limited multiplexing capability, limited applications Limited to single-target detection, Qualitative rather than quantitative	Broughton et al., 2020; Tsou et al., 2019; Xu et al., 2023 Kwon & Shin, 2021
HOLMES	Cas12b	Fluorescence-based detection via Cas12a cleavage	Japanese encephalitis virus (JEV), Pseudorabies virus (PRV)	High sensitivity and specificity, rapid detection, simplicity, POC use	Limited detection sensitivity for low-abundance targets, Potential cross-reactivity with closely related sequences.	Shiyuan et al., 2018

Table 3 (continued)

Summary of nucleic acid detection systems based on CRISPR-Cas.

System name	Effector	Detection method	Target detection	Advantages	Limitations	References
HOLMESv2	Cas12b	Fluorescence detection via Cas12b collateral cleavage	Identification of circular RNA, mRNA splicing and RNA viruses, one step DNA quantification, SNP genotyping, and quantification of DNA methylation	Improved sensitivity and specificity over the original HOLMES system.	Potential limitations in detecting certain target sequences, requires primer optimization	Li et al., 2019
Cas 13a based-assay	Cas 13a	Fluorescence detection	*Klebsiella pneumoniae* (bla_KPC)	Flexible platform, high sensitivity	Collateral cleavage may interfere in complex samples	Cao et al., 2024
SHERLOCK	Cas13a (LWCas 13a)	Lateral flow strip	Dengue virus (DENV), zika virus (ZIKV)	High sensitivity and specificity, single reaction for multiple targets. Portable and field-deployable, simplicity	Increased risk of surface contamination with pre-amplification reaction	
			Escherichia coli (bla_CTX-M)	Portable, label-free, reliable specificity		Gootenberg et al., 2017
SHERLOCK + HUDSON	Cas13a (LwCas 13a)	Combined sample prep (HUDSON) + SHERLOCK fluorescence/lateral flow using Cas13	Dengue virus (DENV), zika virus (ZIKV)	Enables detection directly from body fluids	Increased risk of surface contamination with pre-amplification reaction	Freije et al., 2019
SHERLOCKv2	Cas13b	Enhanced multiplexed detection via Cas13b and lateral flow	Dengue virus (DENV), zika virus (ZIKV), SARS-CoV-2	Improved sensitivity and specificity over the original SHERLOCK system.	More difficult to optimize	Myhrvold et al., 2018
DETECTR	Cas14 (Cas12f)	Phosphorothioate (PT)	ssDNA virus	Ability to detect ssDNA virus	Limited application	Harrington et al., 2018

CONCLUSION

In summary, the CRISPR-Cas system stands at the forefront of next-generation diagnostics, offering unparalleled specificity, sensitivity, and robustness for pathogen detection. This review outlined the evolution, classification, and multifaceted roles of CRISPR-Cas systems in both promoting and inhibiting bacterial resistance and pathogenicity. Although significant progress has been made, important challenges remain in optimizing these systems. Addressing these hurdles will be crucial for advancing CRISPR-based diagnostics. Continued innovation and research are essential to fully harness the potential of CRISPR-Cas technologies, paving the way for more effective, rapid, and accessible pathogen detection strategies that can strengthen global health responses.

LIST OF ABBREVIATIONS

CRISPR	Clustered regularly interspaced short palindromic repeats
Cas protein	Cas-associated proteins
AMR	Antimicrobial resistance
PAM	Protospacer adjacent motif
crRNAs	CRISPR RNAs
RNAP	RNA polymerase
SHERLOCK	Specific high-sensitivity enzymatic reporter unlocking
ICEs	Integrative and conjugative elements
HGT	Horizontal genetic transfer
MGEs	Mobile genetic elements
PCR	Polymerase chain reaction
NASBA	Nucleic acid sequence-based amplification
nPCR	Nested polymerase chain reaction
mPCR	Multiplex polymerase chain reaction
rt-PCR	Reverse-transcriptase PCR
LAMP	Loop-mediated isothermal amplification
NASBACC	Nucleic acid-sequence based amplification CRISPR cleavage
sgRNA	Single guide RNA
EXPAR	Exponential amplification reaction
SDA	Strand displacement amplification
RCA	Rolling circle amplification
FLASH	Finding low abundance sequences of hybridization
DETECTR	DNA endonuclease targeted CRISPR trans-reporter
HOLMES	One-hour low-cost multipurpose highly efficient system
SNP	Single nucleotide polymorphism
HUDSON	Heating unextracted diagnostic samples to obliterate nucleases

AUTHOR CONTRIBUTIONS

Pui Yuei Lee established the review idea and the article structure, prepared the figures and tables. Christina Shook Cheng Chong reviewed article structure and prepared the table. Mee Hoong See, Lee Lee Lai, Paul Michels, Choon Jiat Lee, Rukumani Devi Velayuthan, Yoke Kiet Doon, Yong Hui Tan, Yin Yin Lau, Sze Wan Poong, Phaik Eem Lim and Crystale Siew Ying Lim improved the final manuscript, figures and tables. All authors read and approved the manuscript.

ETHICS APPROVAL

Not applicable.

FUNDING

The study was funded by the Malaysian Ministry of Higher Education through the Fundamental Research Grant Scheme (FRGS/1/2021/STG01/UCSI/03/1).

CONFLICT OF INTEREST

All authors declare no competing interests.

ACKNOWLEDGEMENTS

The study was funded by the Malaysian Ministry of Higher Education through the Fundamental Research Grant Scheme (FRGS/1/2021/STG01/UCSI/03/1).

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Citation:
Lee, P. Y., Chong, C. S. C., See, M. H., Lai, L. L., Michels, P., Lee, C. J., Velayuthan, R. D., Doon, Y. K., Tan, Y. H., Lau, Y. Y., Poong, S. W., Lim, P. E., & Lim, C. S. Y. (2026). CRISPR: An overview of CRISPR-Cas systems and their diagnostic methods for pathogen detection. *Life Sciences, Medicine and Biomedicine*, *10*(1).
https://doi.org/10.28916/lsmb.10.1.2026.232

Life Sciences, Medicine and Biomedicine
ISSN: 2600-7207