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Revolutionary Advancements in Pathogen Detection Technologies Based on Crispr-Cas Systems

Xinye Li [1], Huan Li [2], Shaowen Zheng [3], Lei Zhang [4], Yongkun Fu [5], Yun-mu Zhang [6]*
[1-6] College of Food and Biology, Jingchu University of Technology, Jingmen, Hubei, China
[6] Hubei Engineering Research Center for Specialty Flowers Biological Breeding, Jingchu University of Technology, Jingmen, Hubei, China

* Corresponding Author: **Yun-mu Zhang**

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Abstract

In the past several decades, pathogens have emerged as significant threats to both plant production and human security. The rapid and accurate detection of pathogens is essential for effective disease management, precise therapeutic interventions, and timely remediation efforts. The CRISPR-Cas system, which belongs to the microbial defense systems, distinguishes itself through its exceptional adaptability–requiring only a modification in the crRNA sequence to target various genetic elements–along with its unparalleled precision capable of single-base resolution. Its sensitivity extends to attomolar concentrations, and it maintains programmable, precise while being cost-effective. These features of CRISPR-Cas systems collectively surpass the limitations inherent in traditional molecular diagnostic techniques, positioning the CRISPR-Cas system as a pioneer in the next generation of pathogen detection technologies. In this review, we summarized the novel applications of CRISPR-Cas based technologies for nucleic acid detection, its advantages and disadvantages, as well as some potential challenge, and looked forward to the future development of various systems. These provide important considerations for the improvement of CRISPR-Cas based nucleic acid detection.

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1. Introduction

Infectious diseases caused by pathogens are one of the significant dangers to public health [1]. The variety of pathogens is extensive, with rapid mutations and strong infection capacity. Hence, pathogen-caused infectious diseases severely damage public interest, and even affect social stability and economic development. Therefore, early, rapid, and accurate detection is an important prerequisite for monitoring, preventing, and controlling pathogens [2]. In 2017, the World Health Organization (WHO) published standards for pathogen detection methods, requiring that these methods be affordable, highly sensitive and specific, easy to operate, rapid and stable [3]. Therefore, there is an urgent need to develop more inexpensive, sensitive, simple, and rapid new detection platforms to achieve early detection and control of diseases [4].

Traditional pathogen detection technologies include isolation and culture identification, enzyme-linked immunosorbent assay (ELISA)-based immunological diagnostic techniques, and molecular biological diagnostic techniques based on nucleic acids. Among them, isolation and culture identification have long detection cycles, while ELISA-based immunological diagnostic techniques and molecular biological diagnostic techniques based on nucleic acids all have high detection costs, complicated operation processes, long time consumption, and require highly skilled professionals, making them unsuitable for rapid detection for temporary scene [5, 6]. Since 2012, the emergence of the CRISPR-Cas (clustered regularly interspaced short palindromic repeats-associated proteins) systems have brought about a technological revolution in life sciences. CRISPR-Cas systems can Precisely recognize and cut specific DNA and RNA sequences, demonstrating its powerful diagnostic capability

For nucleic acid detection [7]. Detection technologies developed based on CRISPR-Cas systems have the advantages of high sensitivity, strong specificity (single-base resolution), high flexibility, low cost, and rapid stability, breaking through the limitations of various traditional methods and showing great potential in rapid on-site detection of pathogens [2]. This review aims to introduce the basic principles of the CRISPR-Cas system, summarize the current application status of Cas9, Cas12, Cas13 and Cas114 proteins in related pathogen detections, and provide new ideas for establishing novel pathogen detection platforms.

2. CRISPR-Cas9 technology for pathogen detection

In the CRISPR-Cas9 system, the invaded exogenous bacteria DNA initiates transcription of the CRISPR sequence to produce tracrRNA and pre-crRNA. The pre-crRNA is processed into mature crRNA by ribonuclease III, then the crRNA binds with the tracrRNA to form a single-molecule guide RNA (single-guide RNA, sgRNA). sgRNA binds to the target DNA by guiding Cas9 protein to recognize the protospacer adjacent motif (PAM) at the 3' end of exogenous DNA and unwinds the DNA double helix. Hence, Cas9 protein containing HNH and RuvC-like nuclease domains cleaves both the complementary and non-complementary DNA strands, resulting in a double-strand break (DSB) at targeted locus. The DSB triggers two repair mechanisms: non-homologous end joining (NHEJ) and homology-directed repair (HDR). These mechanisms facilitate following genome editing [8, 9].

CRISPR-Cas9 technology was applied in nucleic acid detection firstly in 2016. By combining with the nuclease acid sequence-based amplification (NASBA) with CRISPR-Cas9 system, Collins *et al*. developed a detection system capable of identifying different subtypes of Zika virus (ZIKV) [10]. The system first used NASBA to reverse transcribe and amplify RNA into dsDNA, and then the dsDNA product could be detected by CRISPR-Cas9 system according to the specific sgRNA designed for different subtypes of ZIKA. The detection result could be read through the color change by naked eyes [10]. Subsequently, Wang *et al*. combined the CRISPR-Cas9 system with a lateral flow nucleic acid detection device, developing a CRISPR-Cas9-mediated lateral flow nucleic acid assay (CASLFA), which could detect Listeria monocytogenes within one hour [11]. Besides, Sun et.al utilized the Cas9-sgRNA binary complex to recognize and cut target sequences, triggering strand displacement amplification and rolling circle amplification, establishing a fluorescent detection method for Escherichia coli O157: H7 [12].

Some detection methods have also been developed by modifying Cas9. When mutations were introduced in both HNH and RuvC-like nuclease domains, deactivated Cas9 (dCas9) losing of double-strand DNA shearing ability but retaining its high binding specificity was obtaine. Zhang *et al*. established an in vitro detection system for detecting Mycobacterium tuberculosis [13]. Kim *et al*. combined the CRISPR-Cas9 system with surface-enhanced raman scattering (SERS) to detect multidrug-resistant bacteria [14]. Guk *et al*. incorporated DNA fluorescent in situ hybridization technology (FISH) with the CRISPR-Cas9 system, constructing DNA-FISH system based on dCas9/sgRNA-SYBR Green I for successful detection of methicillin-resistant Staphylococcus aureus (MRSA) [15].

3. CRISPR-Cas12 technology for pathogen detection

In 2015, Zhang *et al*. discovered a class II V type CRISPR effector protein CRISPR-Cas12a (Cpf1) [16]. CRISPR-Cas12a is an enzyme that binds and cleaves double-stranded DNA. Different from CRISPR-Cas9, CRISPR-Cas12a recognizes 5’ T-rich PAM for target binding and cleavage, and besides the programmable on-target cleavage activity of target dsDNA (cis-cleavage), CRISPR-Cas12a also has the promiscuous cleavage activity of collateral ssDNAs (trans-cleavage) which is triggered by its cis-cleavage [17]. In fact, in addition to the Cas12 system, both Cas13 and Cas14 exhibit the same collateral activity. Consequently, Cas12, Cas13, Cas14 have been developed into nucleic acid detection tools that are distinct from Cas9.

Chen *et al*. coupled recombinase polymerase amplification (RPA) with CRISPR-Cas12a to develop DETECTR (DNA endonuclease targeted CRISPR trans reporter). The DETECTR system can detect the human papillomavirus (HPV) with attomolar (aM) sensitivity and can distinguish between the two subtypes, HPV16 and HPV18[18]. Li *et al*. developed the HOLMES (one-HOur Low-cost Multipurpose Highly Efficient System, HOLMES) technology based on the property of Cas12a/crRNA/target DNA ternary complex to arbitrarily cleave non-targeted ssDNA [19]. In the detection of DNA viruses such as pseudorabies virus (PRV), the HOLMES system can achieve a detection sensitivity of 1 to 10 aM. Similarly, it can sensitively detect RNA viruses like Japanese encephalitis virus. Nowadays, many pathogen detection methods based on the CRISPR-Cas12a system combined with PCR have been established. Li *et al*. developed a fluorescence detection platform Cas12aFDet (CRISPR-Cas12a-based fluorescence detection platform) based on the CRISPR-Cas12a system. This system achieved the specific detection of Listeria monocytogenes serotype 4c within 15 minutes, and the detection sensitivity was able to achieve 0.64×10^{-18} mol/L when coupled with recombinase aided amplification method [20].

The CRISPR-Cas12b (C2c1) protein, which belongs to Class II CRISPR systems, also possesses trans-cleavage activity. Except for HOLMES developed in 2018, Li *et al*. combined the ssDNA trans-cleavage activity of Cas12b with loop-mediated isothermal amplification (LAMP) to develop HOLMESv2 [21]. In HOLMESv2, LAMP amplification and Cas12b trans-cleavage are integrated into a one-step system at a constant temperature, reducing the temperature requirements during the detection process, thus greatly facilitating nucleic acid testing. Teng *et al*. developed a Cas12b-mediated DNA detection system (Cas12b-mediated DNA detection, CDetection) [22]. CDetection system utilized the optimized tgRNA (tuned guide RNA, tgRNA) to distinguish single bases and can be used for the detection of trace amounts of DNA.

4. CRISPR-Cas13 technology for pathogen detection

Unlike the CRISPR/Cas12 system, the CRISPR/Cas13 is an RNA-guided RNA targeting system [23]. CRISPR/Cas13 systems include Cas13a, Cas13b, Cas13c, etc., which can generate specific cleavage sites on RNA regions with specific bases. Taking Cas13a as an example, studies have shown that Cas13a has two lobes, REC and NUC. The NUC lobe contains two HEPN domains, a Helical-2 domain, and a linker domain connecting the two HEPN domains. The two HEPN domains form the active region of Cas13a for cleaving target RNA. The crRNA recognizes and binds to the complementary target RNA to form a double-stranded RNA, causing conformational changes in crRNA and Cas13a protein, prompting the two HEPN domains to move closer together and thereby activating the Cas13a protein. Cas13a activated by crRNA and target RNA exhibits powerful collateral cleavage activity of ssRNA, providing it with excellent signal amplification capabilities.

Gootenberg *et al*. utilized the collateral cleavage activity of Cas13a to establish the first CRISPR/Cas biosensing system based on LwCas13a—SHERLOCK (specific high sensitivity enzyme reporter unlocks, SHERLOCK), which has been successfully applied to rapid detection of pathogens such as the novel coronavirus, norovirus, and dengue virus [23]. By combining RPA technology, SHERLOCK achieved single-nucleotide resolution detection of target nucleic acids at the level of 10^{-18} mol/L. To achieve simultaneously detect of multiple pathogens, the team employed different cleavage preferences of effector proteins like Cas12a, Cas13a, and Cas13b, developing the SHERLOCKv2. By screening four different Cas effector proteins to cleave their respective specific fluorescent reporter molecules, up to four different types of viruses can be detected simultaneously [24]. Additionally, coupling CRISPR Csm6 with Cas13a enhanced the signal by 3.5 times. Following that, Myhrvold *et al*. established HUDSON (Heating Unextracted Diagnostic Samples to Obliterate Nucleases) by combining SHERLOCK with heat treatment to directly conduct viral nucleic acid detection in clinical samples without DNA/RNA extraction and purification processes, greatly simplifying the procedure and facilitating the practical application of this technology [25].

5. CRISPR-Cas14 technology for pathogen detection

Cas14 stands as the most diminutive fully functional CRISPR nuclease (400-700 aa) unearthed to this day. Same as Cas12, Cas14 is able to be guided by sgRNA to seek and cut target ssDNA with accurate location. Different from Cas12, Cas14 does not depend on a PAM site for target recognition, making it more compatible in terms of detection site selection [26]. Currently, Cas14 nuclease has been applied in multiple research fields, such as the diagnosis of infectious viruses and bacteria, detection of cancer cells, and differentiation of SNPs.

Doudna *et al*. utilized Cas14a to detect the human E3 ubiquitin protein ligase 2 gene and compared it with the DETECTR system based on Cas12[26]. In this study, Cas14a recognized blue-eyed SNP while Cas12 failed. Therefore, Cas14a is more suitable for clinical pathogen detection and high-fidelity discrimination of single-base mutations compared to Cas12. Song *et al*. developed a universal bacterial nucleic acid diagnostic platform based on the trans-cleavage activity of Cas14a. Combining Tag-specific primer extension (TSPE) with the cleavage activity of Cas14a, this diagnostic platform achieved detection limit of 1aM or single cell, applicable to the detection of six different pathogenic bacteria [27]. Meng *et al*. reported an isothermal amplification strategy based on Cas14, named integrating competitive annealing mediated isothermal amplification (CAMP) - mediated CRISPR-Cas14a isothermal detection platform (CCIP). CCIP used CAMP to produce abundant ssDNA target sites, then Cas14a would utilize its trans-cleavage activity to release a lots of detection signals that can be observed by naked eyes with high specificity and sensitivity of 10^2 aM. Hence, CCIP is a promising technology for practical pathogen diagnosis [28].

6. Challenges and Prospects of CRISPR-Cas pathogen diagnosis technology

The CRISPR-Cas systems can accurately recognize specific DNA or RNA sequences and distinguish single-base differences. It performs well in detecting unique sequences of specific pathogens, effectively avoiding misdiagnosis. Combining with nucleic acid isothermal amplification techniques, such as RPA and LAMP, it can detect low-abundance nucleic acids. Hence, CRISPR-Cas based detection methods are capable to detect extremely small amounts of target nucleic acids in samples like blood, saliva, etc. In particular, CRISPR nucleic acid detection methods do not require complex thermal cycling processes. The reaction can be carried out under isothermal conditions and completed within half an hour or even less, greatly shortening the detection time. Compared with traditional nucleic acid detection techniques such as PCR, the operation process of CRISPR nucleic acid detection is relatively simplified. It has lower requirements for experimental equipment and the professional skills of operators, making it easier to promote and apply. Hence, it is expected to achieve point-of-care testing. By designing multiple different crRNAs, it can detect multiple pathogens or multiple gene loci of the same pathogen simultaneously, which helps to improve detection efficiency and accuracy and reduce detection costs.

Currently, although CRISPR technology has many advantages in nucleic acid detection, pathogen diagnosis technologies based on the CRISPR-Cas system still face many challenges. Off-target cleavage may still occur in some cases, resulting in false-positive results. Besides, when detecting complex samples (such as blood, tissues, etc.), some components in the samples may inhibit the activity of Cas proteins or affect the detection signal, thus interfering with the accuracy of the detection results. Moreover, most CRISPR-Cas based detection platforms can only perform qualitative detection of a single pathogen, making high-throughput rapid and quantitative detection challenging. To address these limitations, building a high-throughput and quantitative rapid detection platform based on the CRISPR-Cas system may be a future research focus. In summary, through continuous optimization and improvement of the CRISPR-Cas technology for pathogen detection platforms, it will show great potential in pathogen detection and disease prevention and diagnosis.

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