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Research progress of Class 2 CRISPR-Cas system in nucleic acid detection of animal pathogens

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KEYWORDS

CRISPR-Cas, nucleic acid detection, diagnosis, livestock

HIGHLIGHTS

- Novel overview of Class 2 CRISPR-Cas systems for veterinary diagnostics.
- Comparative analysis of Cas9, Cas12, and Cas13 detection platforms.
- Integration with isothermal amplification enables rapid, ultrasensitive assays.
- Multiplex readouts improve on-site applicability and diagnostic accuracy.
- Future directions highlight amplification-free and field-deployable CRISPR tools.

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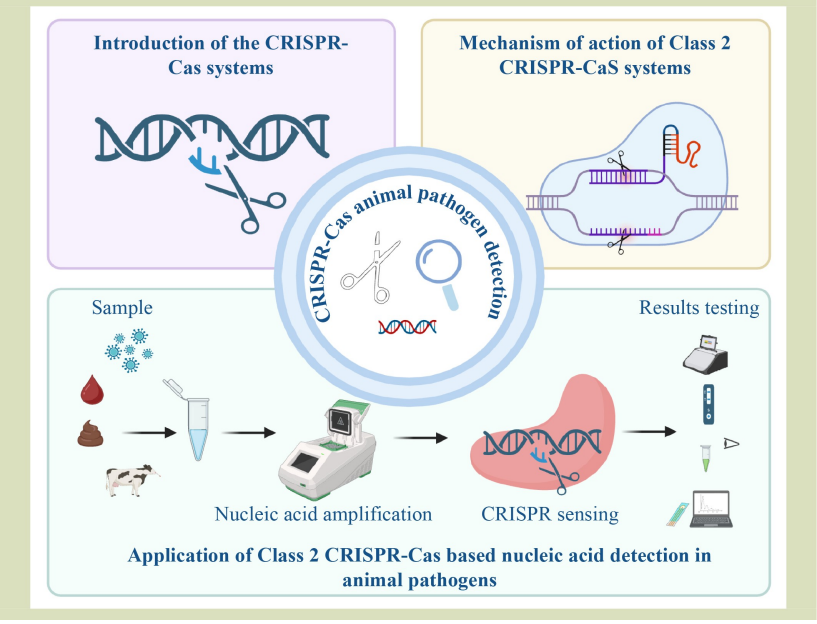
GRAPHICAL ABSTRACT



ABSTRACT

The rapid spread of animal diseases and the evolution of associated pathogens underscore the urgent need for improved diagnostic techniques. Established nucleic acid detection methods typically rely on expensive and complex machinery, which requires specialized expertise and is time-consuming to operate. As a result, these methods are not well-suited for the monitoring and preliminary screening of epidemics in highly-intensive livestock operations. Therefore, there is a pressing need for the development of on-site rapid nucleic acid detection technologies that offer both high sensitivity and specificity. The clustered regularly interspaced short palindromic repeats and associated proteins (CRISPR-Cas) system is notable for its simplicity, precision and high-efficiency gene-editing capabilities. Recent investigations into CRISPR-Cas-based nucleic acid detection methods have demonstrated considerable potential for advancing diagnostic technology in this field. This paper provides a comprehensive review of CRISPR-Cas-based nucleic acid

detection principles and their application in diagnosing animal diseases. It aims to serve as a valuable reference for researchers and practitioners involved in the development and implementation of CRISPR-Cas technologies for animal pathogen detection.

1 Introduction

The occurrence of large-scale epidemic diseases, caused by pathogens such as bacteria[1–3], viruses[4–8] and parasites[9–11], has a significant impact on livestock. These diseases adversely affect the health of animals, which in turn hampers the sustainable development of animal husbandry and leads to significant economic losses in agricultural industry. The production methods used on modern highly-intensive farms are particularly susceptible to the rapid spread of diseases. This susceptibility facilitates the mutation and evolution of pathogens, making the detection and prevention of such diseases an exceptionally complex challenge. Current pathogen detection techniques, including standard pathogen isolation and culture, enzyme-linked immunosorbent assay, indirect immunofluorescence assay, and general polymerase chain reaction (PCR), often depend on large, costly instruments and specialized personnel. Additionally, these methods are constrained by both cost and space limitations and have significant drawbacks[12,13]. The advent of isothermal amplification technology has rendered the use of expensive and complex instrumentation unnecessary, has a user-friendly interface, and can rapidly detect pathogens[14]. Nevertheless, this approach necessitates the availability of high-quality sample templates, is susceptible to non-specific amplification issues, and offers less stability and specificity than PCR[15]. The complex of clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas) can accurately recognize and cleave specific DNA or RNA sequences[16]. Building on this property, the CRISPR-Cas system, while widely recognized as the technology of choice for gene editing[17–19], has also been developed as an innovative tool for nucleic acid detection. By combining CRISPR-Cas with other technologies, it enables more convenient and accurate detection. Compared to end-point PCR-based detection methods, CRISPR-Cas-based nucleic acid detection technologies has superior sensitivity and resolution when combined with suitable amplification strategies. Also, these methods obviate the need for complex instrumentation, expensive reagents, and specialized personnel, representing a substantial advancement in cost-efficiency. When integrated with lateral flow assays or biosensors, the detection results can be interpreted either by direct visual examination or through portable devices, significantly reducing the overall expense and enhancing their suitability for point-of-care applications. This paper provides an overview of the classification, mechanism of action and current applications of CRISPR-Cas-based assays in the detection of animal diseases, and offers insights into their future prospects.

2 Structure of CRISPR-Cas systems

The CRISPR-Cas system consists of three primary components: the CRISPR array, the leader sequence and the Cas genes[20–22]. The CRISPR array is made up of highly conserved, discontinuous repeats[23] and spacers[24,25].Cas proteins encoded by cas genes function as effector nucleases essential for cleaving exogenous genes and acquiring gene fragments[16,26,27]. According to the current classification, the CRISPR-Cas system is divided into two main classes and six subtypes, each further categorized into several subtypes[28]. Types I, III and IV CRISPR-Cas systems, collectively known as Class 1 systems, are found in both bacteria and archaea. These systems require multiple Cas proteins to form a Cas-crRNA complex in order to function. In contrast, Class 2 systems, which include Types II, V and VI, are predominantly found in bacteria and rely on a single Cas protein to associate with crRNA, facilitating nuclease activity[29,30]. The simpler structural composition of Class 2 systems makes them more amenable to development and application in biotechnology, leading to their widespread use in fields such as animal and plant science, microbiology and medicine[31–34].

3 Discovery and mechanism of action of Class 2 CRISPR-Cas systems

Since the immune mechanism of the CRISPR-Cas9 system was

elucidated[35], CRISPR has rapidly become one of the most prominent molecular biotechnologies[36]. In 2015, Cas12 was discovered[37]. In 2016, the CRISPR-Cas9 system was first applied to nucleic acid detection technologies[38]. In June of the same year, Abudayyeh et al.[39] reported the RNA-targeting Cas13a protein (also known as C2c2). Due to its highly specific recognition and editing capabilities, the CRISPR-Cas system has proven to be a powerful tool for nucleic acid detection. The discovery of Cas12 and Cas13 further advanced research into pathogen nucleic acid detection systems, ushering in a new era of study in this field[40–43].

The CRISPR-Cas system provides resistance against various invading foreign genetic elements. Its mechanism of action is primarily divided into three stages: adaptation, expression and interference[44]. When a foreign gene is incorporated into a bacterium for the first time, it is integrated into the CRISPR sequence. Upon subsequent invasion by the same foreign gene, the CRISPR system rapidly generates corresponding recognition sequences that specifically guide Cas nucleases to cleave the foreign gene, thereby providing an immune response (Fig. 1).

The CRISPR-Cas12 system belongs to Class 2, Type V CRISPR systems, with Cas12a, Cas12b and Cas12f being the most extensively studied variants[46]. Cas12a is guided by a single RNA, is smaller in size than Cas9, and can independently process pre-crRNA into mature crRNA without the need for tracrRNA or RNase III[47]. Cas12b (also known as C2c1) is a nuclease that is mediated by both crRNA and tracrRNA or alternatively by a single sgRNA[48]. Cas12f (also known as Cas14) is the smallest RNA-guided nuclease identified to date[49,50]. The CRISPR/Cas12 system specifically cleaves DNA downstream from the PAM site, resulting in double-strand breaks with sticky ends, while also non-specifically cleaving ssDNA (single-stranded DNA) (Fig. 2(b))[49,51,52].

The CRISPR-Cas13 system belongs to the Class 2, Type VI CRISPR system and features two HEPN (higher eukaryotic and prokaryotic nuclease) domains[39], making it a unique CRISPR system that specifically targets RNA[53]. The Cas13 protein catalyzes the maturation of pre-crRNA into mature crRNA[53–55]. When bound to crRNA, the Cas13 protein forms a Cas13-crRNA complex that recognizes a specific protospacer flanking site on the non-target strand, analogous to the PAM sequence recognized by Cas9. Upon binding, Cas13 commences nuclease activity[56]. Similar to Cas12, Cas13 specifically cleaves target RNA while also effecting collateral cleavage activity on nearby RNA (Fig. 2(c)).

4 Application of Class 2 CRISPR-Cas based nucleic acid detection in animal pathogens

The integration of the CRISPR-Cas system with PCR technology, as well as isothermal amplification techniques such as recombinase polymerase amplification (RPA), recombinase-aid amplification (RAA) and loop-mediated isothermal amplification (LAMP), has significantly advanced the field of nucleic acid detection. By amplifying target nucleic acid sequences *in vitro* and coupling these methods with fluorescence, electrochemical signals and commercial test strips, the efficiency of detection is greatly enhanced. This approach simplifies operational procedures and reduces costs. The synergy between the CRISPR-Cas system and these technologies has markedly improved the sensitivity, accuracy, and convenience of pathogen nucleic acid detection.

4.1 Application of CRISPR-Cas9 system in nucleic acid detection of animal pathogens

The CRISPR-Cas9 system, renowned for its extensive applications in gene editing, is increasingly used in the detection of nucleic acids associated with animal pathogens (Table 1). The efficacy of Cas9-based nucleic acid detection hinges primarily on its ability to specifically recognize target sequences. This process involves the design of sgRNAs that are tailored to specific targets, which, upon recognition, activate the cleavage activity of Cas9. This activation results in double-strand breaks in the target DNA. The integration of this cleavage activity with other complementary technologies facilitates the precise detection of nucleic acids, thereby enhancing the diagnostic capabilities in pathogen detection[57,58].

4.1.1 CRISPR-Cas9 in nucleic acid detection

Zhou et al.[59] proposed a CRISPR/Cas9-based fluorescence resonance energy transfer nucleic acid analysis method for detecting *Mycobacterium tuberculosis* by targeting a highly-variable region of *M. tuberculosis* 16S rDNA fragment. In this study, P1 targets the upstream region of the target sequence, whereas P2 covers the downstream region, the target sequence

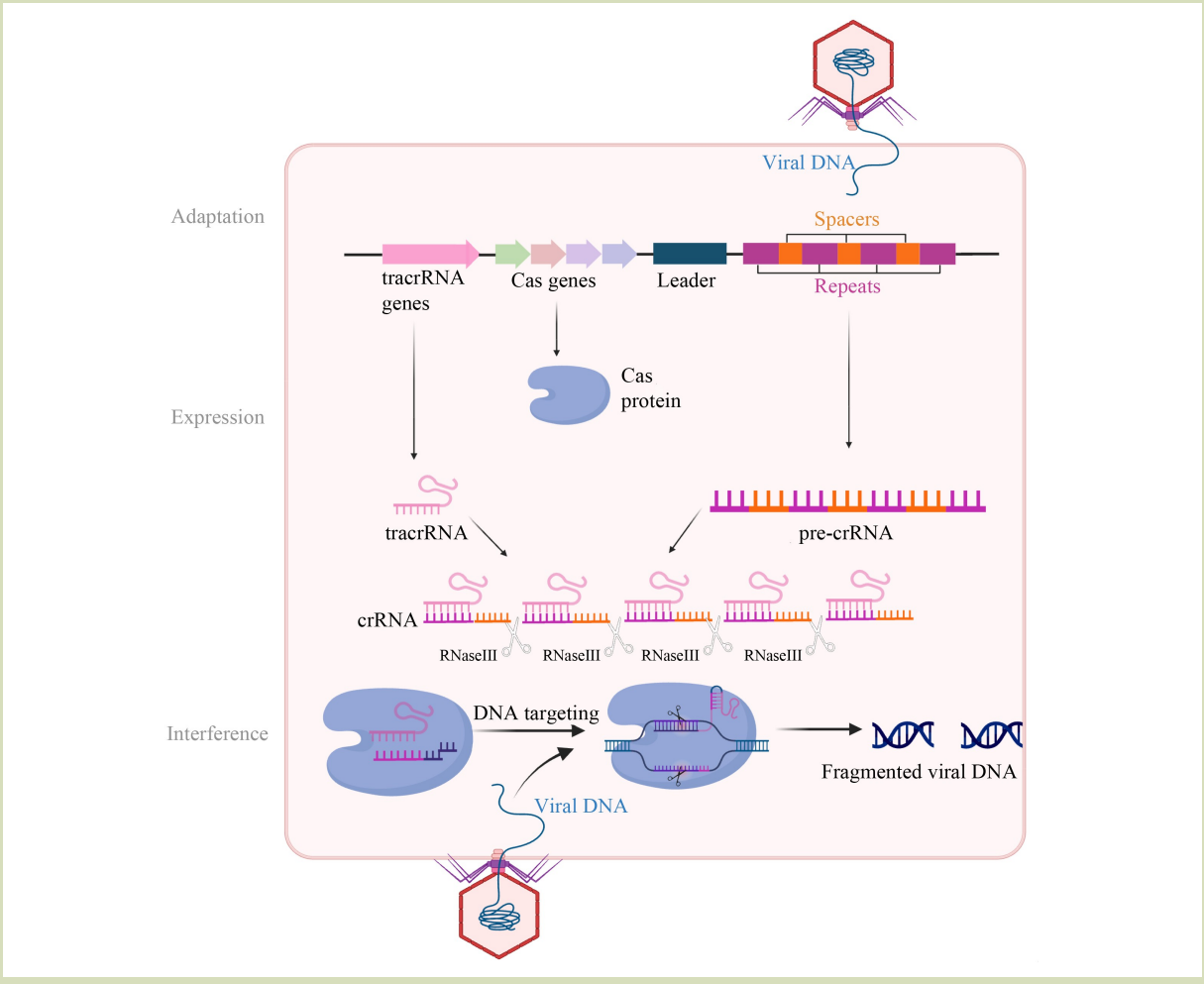


Fig. 1 Mechanism of action of the Type II CRISPR-Cas system (using CRISPR-Cas9 as an example). When foreign genes first enter bacteria, a small fragment of the foreign DNA is stored between two repeat sequences within the CRISPR locus. Upon subsequent infection by the same foreign gene, the CRISPR array is transcribed into precursor crRNA (pre-crRNA). The pre-crRNA is then processed into mature crRNA by ribonucleases or Cas proteins, cleaving at the repeat sequence sites. In the CRISPR-Cas9 system, a tracrRNA sequence located upstream of the Cas genes is transcribed into tracrRNA, which binds to crRNA, forming a complex that guides the Cas9 protein. Finally, the crRNA forms different complexes with Cas proteins, activating the endonuclease activity of Cas proteins to prevent foreign gene integration.

itself and the complementary region of P1. P1 and P2 are conjugated to nanoparticles, specifically UCNPs@SiO_2 and Fe_3O_4@Au, respectively. When P1 and P2 form a complex, the fluorescence of UCNPs is quenched. The introduction of the target sequence and the CRISPR/Cas9 system results in the separation of Fe_3O_4@Au from UCNPs, leading to the restoration of UCNP fluorescence. This enables bacterial detection under 980 nm laser excitation. The use of the CRISPR/Cas9 system allows the sensor to distinguish single-base mismatches within a 10-base range near the PAM region, significantly enhancing the accuracy of nucleic acid detection. Although this method demonstrates high applicability in bacterial detection, further improvements are necessary for rapid on-site detection. In addition, Lin et al.[60] designed a CRISPR/Cas9-based eraser strategy to eliminate false positives in the detection of RNA and DNA viruses. In RT-PCR, sgRNAs are designed to target potential double-stranded DNA (dsDNA) contaminants. During the RT step, the addition of Cas9/sgRNA selectively cleaves the dsDNA contaminants, while RNA templates, single-stranded cDNA and DNA

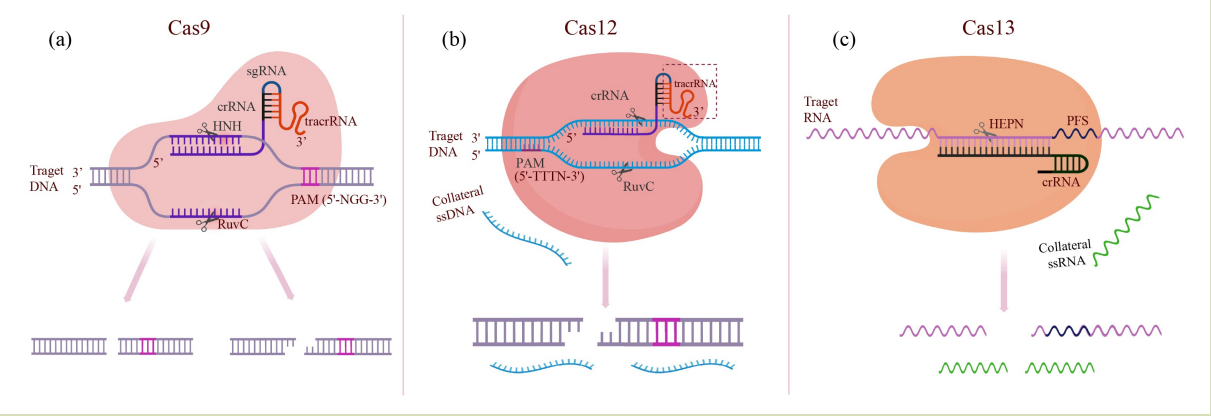


Fig. 2 Mechanisms of action of Cas9, Cas12 and Cas13. (a) The CRISPR-Cas9 system comprises crRNA, tracrRNA (forming the single-guide RNA, sgRNA), and Cas9, which assemble into an effector complex. The Cas9/sgRNA complex recognizes the protospacer adjacent motif (PAM) located at the 3′ end of the foreign DNA. When the target DNA sequence binds complementarily to the crRNA, Cas9 cleaves the DNA a few bases upstream of the PAM site. The RuvC domain of the Cas9 protein is responsible for cleaving the non-target strand, while the HNH domain cleaves the target strand. (b) In the CRISPR-Cas12 system, Cas12a is guided by a single crRNA, whereas Cas12b and Cas12f use an sgRNA formed by crRNA–tracrRNA fusion. Cas12–sgRNA recognizes the PAM on the non-target DNA strand and cleaves the target strand a few bases downstream via its RuvC domain. Additionally, Cas12 has collateral ssDNase activity that indiscriminately cleaves nearby single-stranded DNA. (c) The CRISPR-Cas13 system contains two HEPN (higher eukaryotic and prokaryotic nuclease) domains and is guided by a single crRNA. The Cas13 protein recognizes a protospacer flanking site (PFS) on the non-target strand, which is analogous to the PAM sequence recognized by Cas9 in DNA targeting. Cas13 specifically cleaves the target RNA while also having collateral cleavage activity, leading to the nonspecific degradation of nearby RNA.

Table 1 Applications of Cas9 and its variants in nucleic acid detection

Assay	Target	Target region	Nucleic acid amplification	Readout	LOD	Test duration
FRET nucleic acid analytical method based on CRISPR/Cas9	*Mycobacterium tuberculosis*	16S rDNA		Under 980 nm laser source	20 CFU·mL^{-1}	2 h
CRISPR/Cas9 eraser	ASFV	P72	PCR	LFA		
CASLFA	ASFV	P72	PCR/RPA	LFA	Hundreds of gene copies	30–60 min
CAS-EXPAR	*L. monocytogenes*	With an NCC region	EXPAR	Real-time fluorescent tracking	0.82 amol	< 1 h
Lateral flow strip combined with Cas9 nickase-triggered amplification reaction	*Salmonella typhimurium* and *Escherichia coli*	*E. coli uidA* gene and *S. typhimurium invA* gene	Cas9nAR	Lateral flow strip	100 CFU·mL^{-1}	
A dFnCas9 lateral flow immunoassay	*Leptospira*	*lipL32* gene	PCR	LFA	1 fg·mL^{-1}	< 1 h

Note: CAS-EXPAR, CRISPR/Cas9-triggered exponential amplification reaction; Cas9nAR, Cas9 nickase-based amplification reaction; CASLFA, CRISPR/Cas9-mediated lateral flow nucleic acid assay; CRISPR, clustered regularly interspaced short palindromic repeats; EXPAR, exponential amplification reaction; LFA, lateral flow nucleic acids assay; and RPA, recombinase polymerase amplification.

primers remain unaffected. As the process transitions to PCR thermal cycling, Cas9 nuclease is inactivated at high temperatures. Consequently, only cDNA obtained from RT is amplified during the PCR step, thereby ensuring the fidelity of RT-PCR. However, in the detection of DNA viruses, where DNA contaminants share the same target sequence as the

template DNA, false positives cannot be eliminated through sgRNA design. To address this, a PAM-insertion PCR strategy was introduced. Two additional CC bases were added to the 3′ end of the PCR primers, resulting in an amplified product containing an NGG PAM site, which CRISPR-Cas9 can specifically recognize. By pre-incubating the Cas9/sgRNA with the PCR mixture, contaminant amplicons were selectively removed, thereby eliminating false positive amplification. This approach showed promising applicability in testing 23 suspected ASFV (African swine fever virus)-infected pig serum samples. However, if the contamination source is unknown, sequencing is required before designing new primers and sgRNA. Also, this method still requires expensive equipment and specialized handling systems, making it unsuitable for rapid clinical testing. The lateral flow assay (LFA), a classic rapid detection technique based on antigen-antibody immune reactions, offers portability and quick results. However, its low sensitivity and susceptibility to false positives, along with complex and inefficient hybridization steps, can adversely affect the final detection outcome. Wang et al.[61] integrated Cas9 with the LFA to develop a novel colorimetric biosensing system, CASLFA (CRISPR/Cas9-mediated lateral flow nucleic acid assay), for the detection of *Listeria* and ASFV. This method uses biotin-labeled primers to amplify the target via PCR or isothermal amplification, generating biotinylated products that are specifically recognized by the CRISPR/Cas9 system. The non-target strand is then unwound and released as a single strand. The gold nanoparticle (AuNP)-DNA probe, preloaded in the signal probe hybridization zone, binds to the non-target strand, forming a Cas9-sgRNA-biotin complex. As this complex migrates along the test strip, it is captured by streptavidin at the test line, while the remaining AuNP-DNA probes flow forward and are captured by cDNA probes at the control line. The accumulation of AuNPs on these lines produces visible color bands, indicating the presence of target DNA. Given that this detection system requires AuNP-DNA probes to be designed according to the specific target gene, corresponding test strips must be re-prepared. To address this, Wang et al.[61] introduced an sgRNA2 scaffold by inserting a 15 bp sequence into the stem-loop structure of the sgRNA scaffold, serving as the recognition element for the AuNP-DNA probe. This modification allows different target genes to be detected by simply replacing the sgRNA target recognition region. Under non-laboratory conditions, CASLFA results were consistent with those obtained by RT-PCR, but CASLFA is more convenient and cost-effective, demonstrating significant potential for application in resource-limited settings.

When the PAM sequence is present as a separate DNA oligonucleotide (PAMmer), Cas9 can bind to single-stranded RNA (ssRNA) targets that match the sgRNA sequence. Cas9 can specifically bind to or cleave the RNA targets[62,63]. Based on this, Huang et al.[64] developed a CRISPR/Cas9-triggered exponential amplification reaction strategy (Fig. 3). In this method, the Cas9/sgRNA complex, with the assistance of a PAMmer, recognizes and cleaves the target sequence at specific sites, generating short fragments referred to as X. These X fragments form a complex with the EXPAR template, which contains two regions complementary to the X fragment, separated by nuclease (NEase) recognition sequence. The complex acts as a primer to synthesize dsDNA with a NEase recognition site and two X sequences. The dsDNA is then cleaved by NEase, releasing the X fragment through the action of DNA polymerase. The released X fragment serves as a primer for another EXPAR template, enabling cyclic amplification and the production of a large amount of dsDNA, which is subsequently detected using fluorescent dyes like SYBR Green I. This strategy combines the advantages of CRISPR/Cas9 and exponential amplification, eliminating the need for thermal cycling equipment required in end-point PCR, thereby representing a significant step forward in point-of-care diagnostics. Huang et al.[64] have demonstrated the applicability of this method in detecting total RNA from *Listeria*.

4.1.2 CRISPR-Cas9 nickase in nucleic acid detection

The D10A mutation in Cas9 inactivates the RuvC domain, converting it into a DNA nickase (Cas9n) (Fig. 4(a))[65]. Wang et al.[66] developed a detection technology based on the Cas9 nickase, termed Cas9n-based amplification reaction (Cas9nAR) (Fig. 5). This method leverages the precise recognition and cleavage ability of the sgRNA/Cas9n complex along with the strand displacement activity of Klenow polymerase. The process consists of two consecutive steps. First, two Cas9n/sgRNA complexes cleave the target DNA at two specific sites, displacing ssDNA using DNA polymerase. In the second step, the first primer is added, leading to polymerase amplification and the synthesis of a complementary ssDNA strand. Cas9n then recognizes the newly synthesized ssDNA at its PAM site, cleaving it to generate new ssDNA, which serves as a template for the second prime. This primer then initiates amplification to produce the target strand. The target strand is further cleaved by Cas9nAR, leading to cyclic amplification, which can be monitored via SYBR Green I fluorescence dye. This method allows for

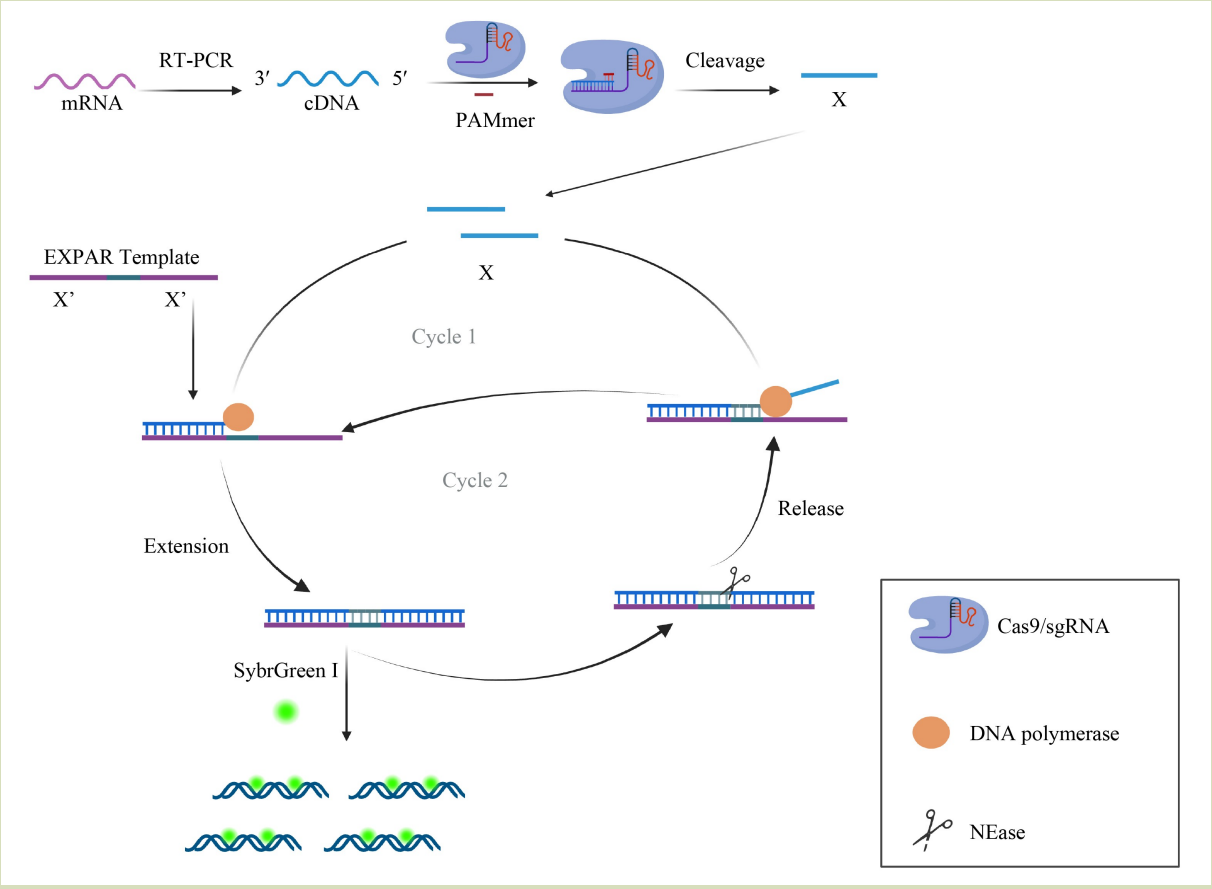


Fig. 3 Mechanism of action of CRISPR/Cas9-triggered exponential amplification reaction. First, a sgRNA is designed to contain three segments: a 20-nt guide sequence, a 30-nt repeat duplex region, and three tracrRNA stem loops. The Cas9 protein forms a complex with the sgRNA and, with the assistance of PAMmers, it recognizes and cleaves the target sequence, resulting in a short ssDNA fragment, X. This fragment is then used as a primer to synthesize double-stranded DNA with the help of the EXPAR template. Subsequently, the double-stranded DNA is cleaved by NEase, releasing the X fragment, which serves as a primer for a cyclic amplification process to generate a large quantity of dsDNA. Real-time fluorescence detection is performed using SybrGreen I during this process.

amplification of target sequences from genomic DNA under isothermal conditions at 37 °C. However, the researchers observed that Cas9nAR had low amplification efficiency. They hypothesized that the stable binding of Cas9n, along with its activated HNH domain, to the cleaved target DNA can hinder DNA polymerase access to the nick site, delaying strand extension. To address this, they performed structure-guided mutagenesis on 16 residues located within four structural domains, promoting the dissociation of the Cas9/sgRNA complex from cleaved DNA. After converting these Cas9 variants into nickases, they identified a variant, Cas9n variant 20 (K848A, N497A, Q695A, Q926A, R1060A and R661A), which had enhanced cleavage activity, naming it fastCas9n. This led to the development of Cas9nAR-v2[67]. In this method, RNA is first reverse transcribed into dsDNA containing two PAM sites. Two different fastCas9n/sgRNA complexes then cleave the dsDNA, producing a ssDNA fragment. This is followed by the cyclic amplification process of Cas9nAR, involving primer binding, extension, nicking, and strand displacement. The system combines reverse transcriptase, DNA polymerase, three sgRNA/Cas9n complexes, two primers and fluorescent intercalating dye, all mixed homogeneously with ribonucleotides and incubated under isothermal conditions. The method was validated by detecting *Salmonella typhimurium* 16S rRNA, *Escherichia coli* O15716S rRNA, synthetic SARS-CoV-2 genes and HIV RNA, demonstrating

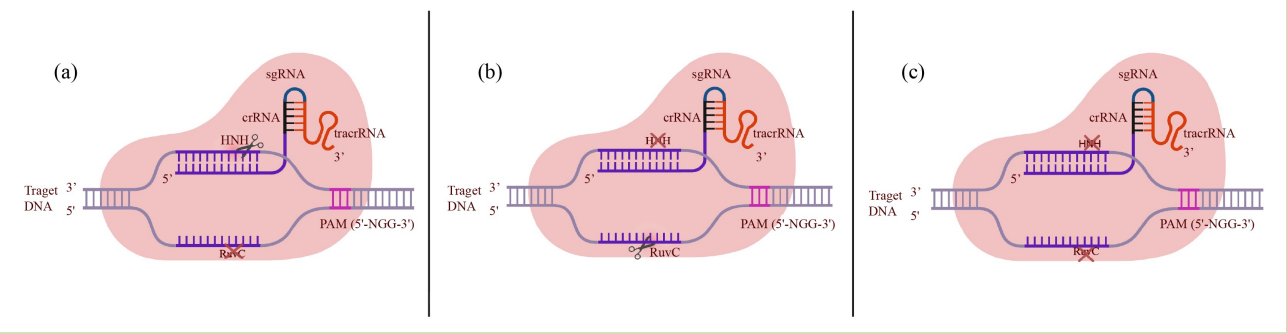


Fig. 4 Mechanism of Cas9 variants. Mutations introduced in Cas9 maintain its ability to bind DNA but inhibit cleavage activity. (a) The D10A mutation inactivates the RuvC domain, which also inhibits cleavage of the coding strand of the target DNA, resulting in a nick that is complementary to the gRNA within the target DNA. (b) The H840A mutation inactivates the HNH domain, preventing it from cleaving the target DNA strand complementary to the sgRNA, thereby creating a nick in the non-complementary or coding strand of the target DNA. (c) When both H840A and D10A mutations occur simultaneously, the HNH and RuvC domains become inactivated, causing the Cas protein to completely lose its cleavage activity while retaining its specific recognition capability.

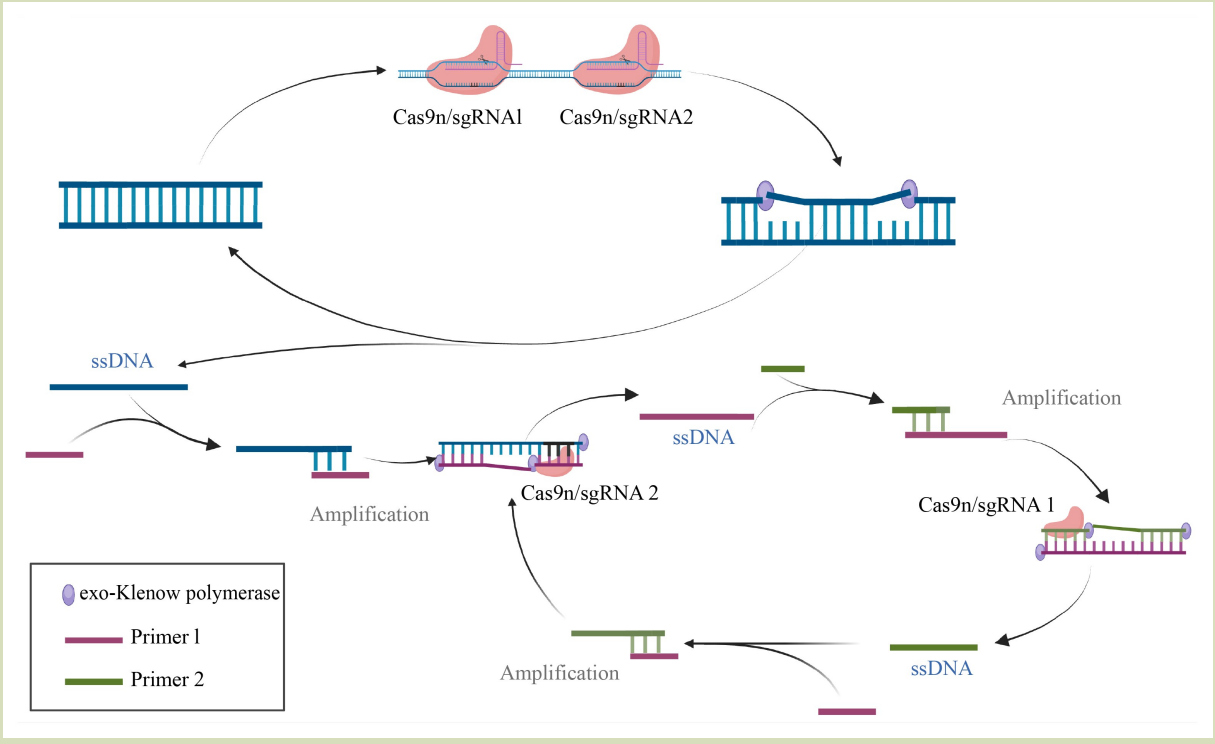


Fig. 5 Mechanism of Action of Cas9nAR. First, two sgRNAs are designed to form a complex with Cas9n, targeting two specific sites on the target DNA for cleavage. This is followed by the use of DNA polymerase to replace the cleaved sites with ssDNA. Then, Primer 1 is added, and under the action of DNA polymerase, a complementary ssDNA strand is generated, resulting in dsDNA. Cas9n/sgRNA recognizes and cleaves to produce new ssDNA, which binds with Primer 2 to form dsDNA. This process continues in cycles. Finally, the results are monitored using SYBR Green I fluorescent dye.

excellent nucleotide mutation discrimination capabilities. In addition, Wang et al.[68] developed a dual foodborne pathogen detection method that combines Cas9nAR with lateral flow test strips. In this approach, primers were labeled with either digoxin/biotin or FITC/biotin. The method successfully amplified *E. coli* and *S. typhimurium* from mixed samples. Detection was achieved by analyzing bands on the lateral flow test strips. This method demonstrated high sensitivity when detecting mixtures of *E. coli* and *S. typhimurium*, highlighting its significant potential for rapid detection of foodborne pathogens.

The H840A mutation in the HNH nuclease domain of Cas9 results in a Cas9 nickase that can recognize double-stranded DNA, but only cleaves the DNA strand that does not interact with the sgRNA (Fig. 4(b))[45]. Therefore, Cas9(H840A) serves as a programmable cutting tool that can substitute for certain endonucleases by targeting and removing specific cutting sites. Sun et al.[69] developed a fluorescence detection method using CRISPR-Cas9(H840A) based on the UiO-66 platform, a metal-organic framework capable of adsorbing ssDNA and emitting fluorescence at specific distances. This method was used for the fluorescence detection of *E. coli* O157. Two Cas9 (H840A)/sgRNA complexes recognize and introduce two nicks in the target strand, allowing exo-Klenow polymerase to extend the 3′ ends at the nick sites and displace the ssDNA. A circular probe is then hybridized to the 3′ end of the ssDNA, and phi29 DNA polymerase synthesizes long copies of the circular probe through rolling circle amplification, producing DNA with repeating sequences. When a DNA probe (FAM) complementary to the ssDNA binds to UiO66, fluorescence is quenched. In contrast, in the presence of long ssDNA, the DNA probe dissociates from UiO66 and hybridizes with the long ssDNA, restoring fluorescence. Therefore, the target DNA is quantified by fluorescence intensity. The method demonstrates enhanced sensitivity and a broad detection range under mild reaction conditions, showing significant potential for applications in bacterial detection, food safety monitoring and clinical diagnosis.

In addition, researchers have mutated the two nuclease domains of Cas9 to inactivate its enzymatic activity while retaining its ability to specifically bind to target proteins (Fig. 4(c))[70]. This nuclease-inactivated Cas9 has been used in diagnosing parasitic diseases. Natarajan et al.[71] developed a dFnCas9-based lateral flow immunoassay for detecting the *Leptospira lipL32* gene. This method uses an FITC-labeled chimeric gRNA (crRNA:tracRNA) chemically modified with a synthetic scaffold to enhance reading stability, forming a complex with dFnCas9 that specifically binds the target sequence. The detection is performed using lateral flow test strips. For quantification, FITC antibodies conjugated with the fluorescent dye AlexaFluor-674 are incorporated into the binding pad of the test strip. This method represents an ideal tool for point-of-care clinical diagnostics.

4.2 Application of CRISPR-Cas12 system in nucleic acid detection of animal pathogens

CRISPR-Cas12 is a member of the CRISPR-Cas system Type V. Cas12a, a subtype commonly used in CRISPR nucleic acid detection, can recognize and cleave target sequences near the PAM site in dsDNA[72]. In 2016, Abudayyeh et al.[39] reported that Cas12a has a unique collateral cleavage activity. Under the guidance of crRNA, Cas12a not only specifically targets and cleaves dsDNA but also has strong non-specific trans-cleavage activity, which degrades any non-target ssDNA. This property has led to the widespread use of the CRISPR-Cas12a system in nucleic acid detection technologies. Aquino-Jarquin G later discovered CRISPR-Cas12f, also known as Cas14, which belongs to the Type V-F CRISPR-Cas system[73]. Cas12f is currently the smallest RNA-mediated Cas protein, about half the size of Cas9, and does not require a PAM site to target ssDNA[49]. The Cas12 system offers several advantages and holds great promise in pathogen nucleic acid detection (Table 2).

4.2.1 Recombinase polymerase amplification combined with CRISPR-Cas12 in nucleic acid detection

RPA is an isothermal amplification technique that can serve as an alternative to PCR. The RPA reaction system includes a single pair of primers and three key enzymes: recombinase, which binds to the oligonucleotide primers; single-strand DNA binding protein (SSB); and strand-displacing DNA polymerase[15]. Integrating RPA with CRISPR can enhance detection sensitivity, as both methods operate effectively within the temperature range of 37–42 °C. This compatibility facilitates their successful integration[74,75]. Fu et al.[76] developed a fluorescence detection method for early ASFV infection using RPA-Cas12a. This method amplifies the target DNA (p72 gene) with RPA, and Cas12a, guided by sgRNA, specifically cleaves the target DNA. The trans-cleavage activity of Cas12a then cuts fluorescence-quenched molecular probes

Table 2 Applications of Cas12 in nucleic acid detection

Assay	Target	Target region	Nucleic acid amplification	Readout	LOD	Testing duration
RPA-Cas12a-fluorescence assay	ASFV	p72	RPA	Visual inspection or Fluorescence	2 copies	30–40 min
DETECTR	ASFV	p72	RPA	Fluorescence	8 copies	< 1 h
DETECTR	*Toxoplasma gondii*	B1	RPA	Fluorometer or LFS	35 copies	< 1 h
DETECTR	*T. gondii*	RE	RPA	Fluorometer or LFS	200–300 copies	< 1 h
RPA-CRISPR/Cas12a	ASFV	KP177R	RPA	Fluorometer or LFD	6.8 copies μL^{-1}	< 30 min
One-pot-RPA-Cas12a	ASFV	B646L	RPA	LFA	3.07 copies μL^{-1}	40 min
One-pot-RPA-Cas12a	CaPV	LSD74	RPA	LFA	1.02 copies μL^{-1}	40 min
Fluorescence and colorimetric analysis based on the RPA-assisted CRISPR/Cas12a strategy	ASFV	B646L	RPA	Naked-eye	20 copies mL^{-1}	< 30 min
Highly sensitive CRISPR/Cas12a-based fluorescence detection	PRRSV	nsp2	RT-RPA	Fluorescent readout	1 copy	< 25 min
RPA-Cas12a-fluorescence assay	LSDV	orf068	RPA	Under UV/LED-blue light	10^2 $TCID_{50}$ mL^{-1}	15 min
RPA-CRISPR/Cas12a assay	*T. gondii*	B1	RPA	Fluorometer or LFS	3.3 copies μL^{-1}	
CORDS	ASFV	p72	RAA	LFS	600 copies μL^{-1}	< 1 h
CORDSv2	ASFV	p72	RPA	Lateral flow, fluorescence	0.6–6 copies μL^{-1}	1 h
CRISPR/Cas12a-LFD	ASFV	B646L	RAA	LFD	20 copies	1 h
RAA-Cas12a-Tg	*T. gondii*	529 bp-RE	RAA	Fluorescence	1 fmol·L^{-1}	1 h
RAA-CRISPR Cas12a	PRRV	V N and M gene	RAA	blue or UV light or LFS	10 copies μL^{-1}	50 min
LAMP-CRISPR	PCV2	rep	LAMP	Fluorescence	1 copy μL^{-1}	1 h
combination of a CRISPR/Cas14a system	ASFV		PCR	Visual	5 copies μL^{-1}	

Note: CaPV, capripoxvirus; DETECTR, DNA Endonuclease Targeted CRISPR Trans Reporter; RPA, recombinase polymerase amplification; CORDS, Cas12a-based On-site and Rapid Detection System; PCV2, porcine circovirus type 2; RAA, recombinase-aided amplification; LAMP, loop-mediated isothermal amplification; LFD, lateral flow detection; and LFS, lateral flow strip.

(ssDNA with a fluorophore at one end and a quencher at the other). Fluorescence signals are collected, and results are identified either visually or through fluorescence analysis. Compared to CRISPR technology and lateral flow assays[77,78], this method offers greater sensitivity and is particularly effective for early infection detection of ASFV. In 2018, Chen et al.[42] developed the DETECTR (DNA endonuclease-targeted CRISPR trans reporter) system, which combines RPA with LbCas12a (Fig. 6). This system uses RPA to amplify target sequences and incorporates a fluorescence quenching (FQ) molecular probe into the reaction system. Upon recognition of the amplified target sequence, LbCas12a generates non-specific cleavage activity, which separates the fluorescent group from the quencher, resulting in the release of fluorescence signals. This method enhances detection sensitivity to attomolar (amol·L^{-1}) levels. Li et al.[79] developed a method for detecting ASFV using DETECTR with FnCas12a, which has enhanced cleavage capacity, in combination with RPA. This approach demonstrated high specificity and improved efficiency compared to qPCR. Recently, researchers developed a method for detecting *Toxoplasma gondii* infection in blood using DETECTR technology combined with LFA[80]. AuNPs coupled with FITC antibodies were immobilized in the sample binding zone of the test strip, with the detection line labeled with streptavidin and the control line labeled with anti-FITC antibodies. After the reaction system is added, the ssDNA reporter molecule (FITC-TTATTATT-biotin) forms a complex with FITC antibody-conjugated AuNPs. Upon Cas12a

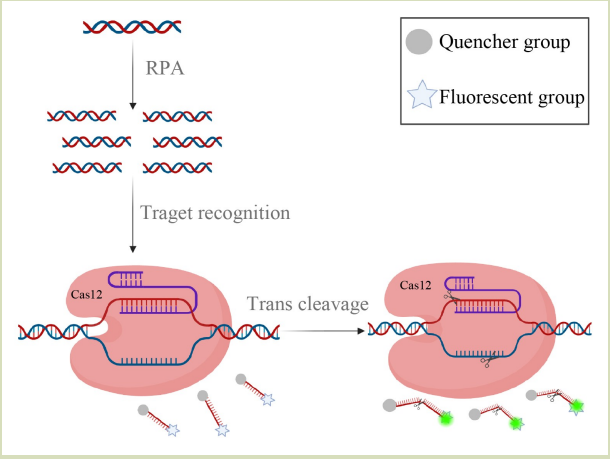


Fig. 6 Schematic of the DETECTR detection system. The DETECTR system begins with the exponential amplification of the target sequence using recombinase polymerase amplification (RPA). Guided by crRNA, the LbCas12a enzyme recognizes and cleaves the dsDNA, activating its non-specific cleavage activity. This leads to the cutting of ssDNA reporter molecules. These reporter molecules contain both a fluorescent group and a quencher group; cleavage separates these groups, releasing a fluorescent signal that indicates the presence and quantity of the target in the sample.

activation, the complex is not captured by the detection line, resulting in the appearance of only the control line. Testing blood samples from infected mice revealed a detection limit of 1.5 copies per μL, with positive results identifiable at early stages of infection. Additionally, Luan et al.[81] developed a CRISPR/Cas12a-based detection method targeting the ASFV KP177R gene, combining it with RPA. This method allows for visual detection through either fluorescence using a fluorescent reporter group or lateral flow strip detection with a biotin-labeled reporter. The assay serves as a valuable tool for the early screening of viruses in the environment or infected pigs. Xiong et al.[82] developed a one-pot RPA-Cas12a platform that integrates RPA and CRISPR-Cas12a. This system combines RPA and Cas12a reagents in a single step by adding them to the top and bottom of a test tube and mixing through centrifugation. This approach effectively mitigates aerosol contamination during the detection process. Also, the study combined the one-pot RPA-Cas12a platform with lateral flow strips for the detection of ASFV and capripoxvirus, making the method more suitable for virus detection in remote mountainous areas. Lin et al.[83] demonstrated that glycerol additives significantly enhance the detection efficiency of the one-pot RPA-CRISPR/Cas12a method, which has been successfully applied for ASFV detection.

Mao et al.[84] developed an advanced system integrating RPA, CRISPR/Cas12a, and magnetic beads (MB)-ssDNA-ALP for precise dual-mode colorimetric and fluorescent detection of ASFV genes. MB-ssDNA-ALP was synthesized through a click reaction between biotin-ssDNA-azide and DBCO-ALP, generating biotin-ssDNA-ALP, which was then conjugated with streptavidin-modified magnetic beads. This method uses RPA to amplify the target DNA, which then hybridizes with crRNA, activating Cas12a to cleave the MB-ssDNA-ALP complex. After magnetic separation, ALP is released from the MB-ssDNA-ALP complex and catalyzes the conversion of p-nitrophenyl phosphate into p-nitrophenol. By introducing quantum dots as potential fluorescent reporters, both fluorescence and colorimetric changes are induced. The integration of colorimetric and fluorescent results enhances the accuracy and sensitivity of the analysis. This method demonstrated excellent performance in detecting clinical samples. Liu et al.[85] designed six types of reporter genes and selected the FAM-BHQ2 ssDNA reporter, which had the strongest fluorescence intensity, as the molecular probe. By combining PCR amplification with the trans-cleavage ability of CRISPR-Cas12a, the detection sensitivity for PRRSV was significantly enhanced. To facilitate on-site detection, researchers used RT-RPA or RPA to amplify the target sequence and established a field fluorescence detection method for PRRSV using the trans-cleavage activity of Cas12a. The method determined that reagents could be added to a single tube for a one-step assay, enabling single-copy detection of PRRSV. Jiang et al.[86] used an RPA-Cas12a fluorescence assay to detect bovine lumpy skin disease virus. Clinical results demonstrated that the diagnostic sensitivity of this method was comparable to that of qPCR, while its specificity exceeded that of both end-point PCR and qPCR. Lei et al.[87] designed a method for detecting *T. gondii* in environmental samples using RPA/Cas12 technology. Their approach involves isolating and extracting *T. gondii* tachyzoites from a liquid solution with a simple glass microfiber filter embedded in paper, eliminating the need for centrifugation equipment. The process begins by isolating DNA from the tachyzoites in a lysate. The DNA sample is added to the freeze-dried RPA mixture at the bottom of the tube for RPA amplification. Then, the tube is inverted to mix the amplicons with the freeze-dried CRISPR/Cas12a reagents, allowing the Cas protein to recognize and cleave the amplicons. This triggers the trans-cleavage of molecular

probes, releasing FAM and BHQ1 or biotin groups. Finally, detection is carried out using either fluorescence or lateral flow strips.

4.2.2 Recombinase-aid amplification combined with CRISPR-Cas12 in nucleic acid detection

RAA is a novel isothermal nucleic acid amplification technique. It uses recombinase, SSB, and primers to form a complex that scans dsDNA. The primers bind to homologous sequences, leading to the unwinding of dsDNA. SSB then stabilizes the ssDNA to prevent re-annealing, allowing DNA polymerase to extend the chain. This process results in amplification of the target nucleic acid. Bai et al.[88] applied the RAA technique combined with Cas12 in a method known as CORDS for detecting ASFV. In their approach, RAA reagents, RAA buffer and Cas12 components were lyophilized and stored in separate tubes. At the time of use, the lyophilized powders were dissolved and samples were added. Detection was performed using lateral flow chromatography paper, with results obtained after a 1 h incubation at 37 °C. Subsequently, CORDSv2 was developed based on the original CORDS system, enabling the RPA and Cas12 mixture to react in a single tube (one-pot)[89]. However, the inherent exonuclease activity of the DNA polymerase in the RPA mixture could lead to non-specific cleavage of the ssDNA probe, interfering with the final results. To address this issue, a new DNA probe, FQ2 (5′ -FAM-TTATT-BHQ1-3′), was designed with optimized length and secondary structure, providing improved stability. To facilitate its application in point-of-care tests, the researchers further developed a compact, portable detection device called hippo-CORDS, a powerful heating and imaging device for on-site rapid detection based on one-pot Cas12a. After adding the sample, the CORDSv2 reaction tube is incubated in the preset 37 °C device for 40 min. Detection is performed by direct visual observation of fluorescence under LED blue light or by taking a photo with a smartphone. Wang et al.[78] developed a CRISPR/Cas12a lateral flow diagnostic method that integrates RAA with LbCas12a and lateral flow test strips. This method amplifies target sequences using RAA technology and then uses the collateral-cut activity of Cas12a to cleave digoxin- and biotin-labeled ssDNA. Results are detected via lateral flow test strips. Ma et al.[90] developed a real-time detection method for *T. gondii* oocysts in soil with a sensitivity reaching the femtomolar level. This method targets the *T. gondii* 529 bp repeat element by designing a specific crRNA and optimizing the RAA reaction (39 °C for 20 min). Cas12a is activated for trans-cleavage of the ssDNA-FQ molecular probe, and fluorescence intensity is observed under UV light or analyzed using a microplate reader to measure fluorescence wavelength. Xu et al.[91] combined Cas12 with RAA to develop a detection method for peste des petits ruminants. The results can be visualized using UV light, blue light or sidestream strips, offering a novel approach for diagnosing that disease based on the N gene (nucleocapsid) and the M gene (matrix).

4.2.3 Other techniques combined with CRISPR-Cas12 in nucleic acid detection

LAMP is a sophisticated isothermal amplification technique that uses four primers, two external and two internal, and a strand-displacing DNA polymerase (Bst DNA polymerase). This method amplifies six specific regions of DNA from the target gene sequence at a constant temperature. Lei et al.[92] developed a rapid, convenient and accurate assay for detecting porcine circovirus type 2 by combining LAMP with CRISPR/Cas12a. This method uses FAM-BHQ1 as a fluorescent reporter, enabling real-time visualization of results under UV light. Ki et al.[93] developed a dual enzyme colorimetric biosensing system that combines the CRISPR/Cas12a system with urease for rapid ASFV detection. This method uses CRISPR-Cas12a to specifically recognize ASFV target sequences, triggering a trans-cleavage activity that cleaves a magnetic bead-anchored urease-conjugated single-stranded oligodeoxynucleotide, thereby releasing urease from the bead. The released urease catalyzes a cascade reaction in the supernatant, hydrolyzing urea and increasing the pH of the solution, which results in a reddish color. This approach enables virus detection without the need for target gene amplification. Single-mode fluorescence or colorimetric assays can be prone to environmental interference, potentially leading to inaccurate results. Zhao et al.[94] developed a cost-effective, highly sensitive colorimetric method for detecting the ASFV gene by combining CRISPR-Cas12f with G4 DNAzyme and a microfluidic paper-based analytical device (μPAD)[95]. Primers modified with phosphorothioate[96], which prevent cleavage by T7 exonuclease, were amplified by PCR. T7 exonuclease then selectively acted on the unmodified strand to produce target ssDNA. After hybridizing with ssDNA, the trans-cleavage activity of the Cas12f complex is triggered. The activated CRISPR/Cas12f system then non-selectively cleaves the G4 DNAzyme, leading to the loss of its peroxidase-like activity, rendering it unable to catalyze the colorimetric substrate. The resulting color change allows for direct visual differentiation of the outcome. Additionally, researchers integrated this method with a μPAD-based colorimetric platform, performing ASFV

detection by mixing the CRISPR/Cas12f system with the G4 DNAzyme complex on hydrophobic wax-patterned microwells. The results were recorded using a smartphone camera and further analyzed with ImageJ software for pixel analysis. The feasibility of the method was validated by detecting ASFV in pig plasma. Wu et al.[97] developed a detection platform based on RT-LAMP and CRISPR/Cas12a to rapidly and accurately differentiate between RHDV1 and RHDV2. To enable portable and visual detection, they used a biotinylated single-stranded DNA (ssDNA) reporter probe (5′ -6-FAM-TTATT-Biotin-3′) in combination with a lateral flow strip. The control line was labeled with streptavidin, while the test line (T-line) was coated with anti-mouse immunoglobulin G antibodies. When the RT-LAMP-Cas12a reaction products were applied to the sample pad, the FAM-labeled end of the ssDNA reporter probe conjugated with AuNPs on the pad. Upon activation of Cas12a trans-cleavage activity, the ssDNA reporter was indiscriminately cleaved. The cleaved FAM-labeled fragments migrated to the T-line, where the immunoglobulin G antibodies captured the AuNP-conjugated fragments, resulting in a visible color signal on the T-line, indicating a positive result.

4.3 Application of CRISPR-Cas13 system in nucleic acid detection of animal pathogens

CRISPR-Cas13 is a component of the Type VI CRISPR-Cas system. In 2016, researchers discovered that Cas13 has incidental-cleavage activity[39]. Specifically, the activation of Cas13 targets and cleaves the substrate RNA, while also non-specifically degrading free single-stranded RNAs present in the environment[57]. The Cas13 system has been widely applied in pathogen nucleic acid detection (Table 3).

In 2017, Gootenberg et al.[40] developed the SHERLOCK (specific high-sensitivity enzymatic reporter unlocking) molecular diagnostic platform based on CRISPR-Cas13a (Fig. 7). This platform uses primers tagged with a T7 promoter to achieve exponential amplification of target nucleic acids via RPA (for DNA detection) or RT-RPA (for RNA detection). The amplified products, containing a T7 promoter, are transcribed into target RNA by T7 RNA polymerase. The Cas13a-crRNA complex then specifically recognizes and cleaves the target RNA and an ssRNA probe labeled with a fluorophore at one end and a quencher at the other. Fluorescent signals generated by probe cleavage are captured for diagnostic purposes. Inspired by this, the combination of RPA/RAA, T7 transcription, and the collateral cleavage activity of CRISPR-Cas13a has been applied to detect canine parvovirus[98], p27 gene of ASFV[99], PCV4 Cap gene[100], PRRSV[101] and BVDV[102]. Additionally, the HUDSON method[103] was developed, utilizing heat and chemical reduction to lyse viruses and inactivate high levels of RNases in body fluids. When combined with SHERLOCK, this method enables direct viral detection from bodily fluids. Subsequently, the team optimized SHERLOCK based on Cas-specific cleavage preferences, developing SHERLOCKv2. This enhanced system incorporated LwaCas13a (which cleaves AU probes), CcaCas13b (which cleaves UC probes), PsmCas13b (which cleaves GA probes), and Cas12a (which recognizes different crRNA sequences and cleaves ssDNA probes distinct from Cas13). This enabled a quadruplex detection system capable of detecting four different target nucleic acids simultaneously. For the first time, they used colloidal gold lateral flow strips to demonstrate CRISPR detection results. The ends of the reporter probes were labeled with FAM (or FITC) and biotin, and detection was achieved using different antibodies (or streptavidin). Also, they introduced an additional CRISPR-associated enzyme, Csm6, to amplify the detection signal, further increasing the sensitivity of SHERLOCK[104]. Ren et al.[105] developed a Cas13-based method for ASFV detection. This approach uses the HUDSON method to remove nucleases from the sample, allowing for direct application of the sample solution to the reaction system[103]. Combined with qPCR, this method is suitable for routine laboratory diagnostics. For field testing, the RPA-CRISPR method with lateral flow chromatography test strips (lateral flow strips) offers enhanced practicality. Zhang et al.[106] used the HUDSON method to replace nucleic acid extraction and combined it with RT-RAA and Cas13a for detecting classical swine fever virus. This approach significantly improves the accuracy of distinguishing between classical swine fever virus strains and vaccines compared to established methods. It can also be integrated with lateral flow chromatography for visual readout, facilitating instrument-free detection from sample processing to result interpretation. Nucleic acid detection using RT-qPCR alone can have low sensitivity and even result in false positives. To address this issue, Zhao et al.[107] proposed a detection method based on RT-PCR combined with RspCas13d, which is significantly smaller than other Cas13 subtypes and does not require a protospacer flanking site[108,109]. This method targets porcine deltacoronavirus by designing PCR primers and crRNA specific to the conserved regions of this virus. RNA is reverse transcribed into cDNA and amplified using RT-qPCR.

Table 3 Applications of Cas13 in nucleic acid detection

Assay	Target	Target region	Nucleic acid amplification	Readout	LOD	Testing duration
SHERLOCK	CPV-2		RPA	Fluorescence	100 amol·L^{-1}	30 min
CRISPR/Cas13a-LFD	ASFV	p72	RAA	Lateral flow strip	10 copies μL^{-1}	< 1 h
RPA-Cas13a-LFD	PCV4	Cap	RPA	Lateral flow strip	1 copy	< 1.5 h
enhanced Cas13a lateral flow detection	PRRSV	M	RPA	Lateral flow, fluorescence	172 copies μL^{-1}	
LwCas13a-based detection system	BVDV	Reported BVDV sequence in 5′UTR conserved region		Fluorescence	10^3 pmol·L^{-1}	
Lateral flow strip-based RPA-CRISPR assay	ASFV	P72	RPA	Lateral flow strip	100 copies μL^{-1}	< 1.5 h
HUDSON-RT-RAA-CRISPR/Cas13a	CSFV	CSFV synthetic RNA templates	RAA	lateral flow strips	300 copies μL^{-1}	
RT-PCR-RspCas13d	PDCoV	The conserved N region	RT-PCR	Fluorescence	4 copies μL^{-1}	30 min
OBServe	ASFV	B646L	multiplex RPA	fluorescent or lateral-flow readouts	1.6 copies μL^{-1}	< 1 h
RPA-based CRISPR-Cas13a detection system	TMUV	NS3	RPA	lateral flow detection	100 copies μL^{-1}	50 min
Visual detection of DTMUV with CRISPR/Cas13	DTMUV	E	RPA	lateral flow and fluorescence	1 copy μL^{-1}	
RT-RAA-Cas13a-LFD	H5-AIV	HA	RAA	lateral flow detection	0.1 copy μL^{-1}	40 min
DRC-LFA	DHAV-3 and NDRV	VP1 and S3	RPA	LFA	10 copies μL^{-1}	35 min

Note: CSFV, classical swine fever virus; NDRV, novel duck reovirus; DHAV-3, duck hepatitis A virus type 3; DRC, dual RPA-CRISPR strategy coupled; HUDSON, heating unextracted diagnostic samples to obliterate nucleases; and SHERLOCK, specific high-sensitivity enzymatic reporter unlocking.

The amplification product is then transcribed into RNA by T7 RNA polymerase and cleaved by the RspCas13d/crRNA complex. Collateral cleavage activity of the Cas protein further cleaves the probe, releasing a fluorescent signal. Compared to standard RT-qPCR, this detection method demonstrates improved specificity and sensitivity. Cas12 and Cas13 are capable of cleaving ssDNA and ssRNA probes, respectively. Wang et al.[110] developed OBServe (orthogonal CRISPR-Cas12b/Cas13a-based dual-gene detection assay that served as a field-deployable tool) for the rapid detection of ASFV and its variants in the field settings. For multiplex RPA, researchers designed primers for the conserved gene B646L (with a T7 RNA polymerase promoter at the 5′ end of the forward primer to enable transcription) and standard primers for the virulence-related gene MGF505-2R (as MGF below). ssRNA probes with 5′-ROX-UUUUUU-BHQ2-3′ are used for Cas13a, and ssDNA probes with 5′-FAM-TTTTT-BHQ1-3′ are used for Cas12b (carboxyfluorescein, FAM; carboxy-X-rhodamine, ROX; black hole quencher 1/2, BHQ1/2) to facilitate in-tube fluorescence reading. In the absence of these two target genes, OBServe has no fluorescence, indicating no ASFV presence. when ASFV with a deletion of the MGF gene is detected, OBServe fluoresced red. For wild-type ASFV, OBServe emits bright yellow fluorescence due to the overlap of green and red signals. This method provides an effective tool for monitoring ASFV and its variants in field environments.

Tambusu virus (TMUV) and avian influenza virus (AIV) have both caused significant economic losses to the poultry industry. He et al.[111] developed a rapid detection method for the conserved NS3 gene in duck TMUV (DTMUV) by combining the CRISPR-Cas13a system with RT-RPA. The target RNA is pre-amplified through RT-RPA, and the resulting amplification product is transcribed into RNA by T7 transcription. This RNA is then detected and cleaved by the Cas13a/crRNA complex, which subsequently cleaves RNA probes, generating amplified fluorescence signals. This method can also be combined with portable lateral flow strips, making it more suitable for on-site detection. Meanwhile, Yin et al.[112] developed a convenient diagnostic method for detecting the

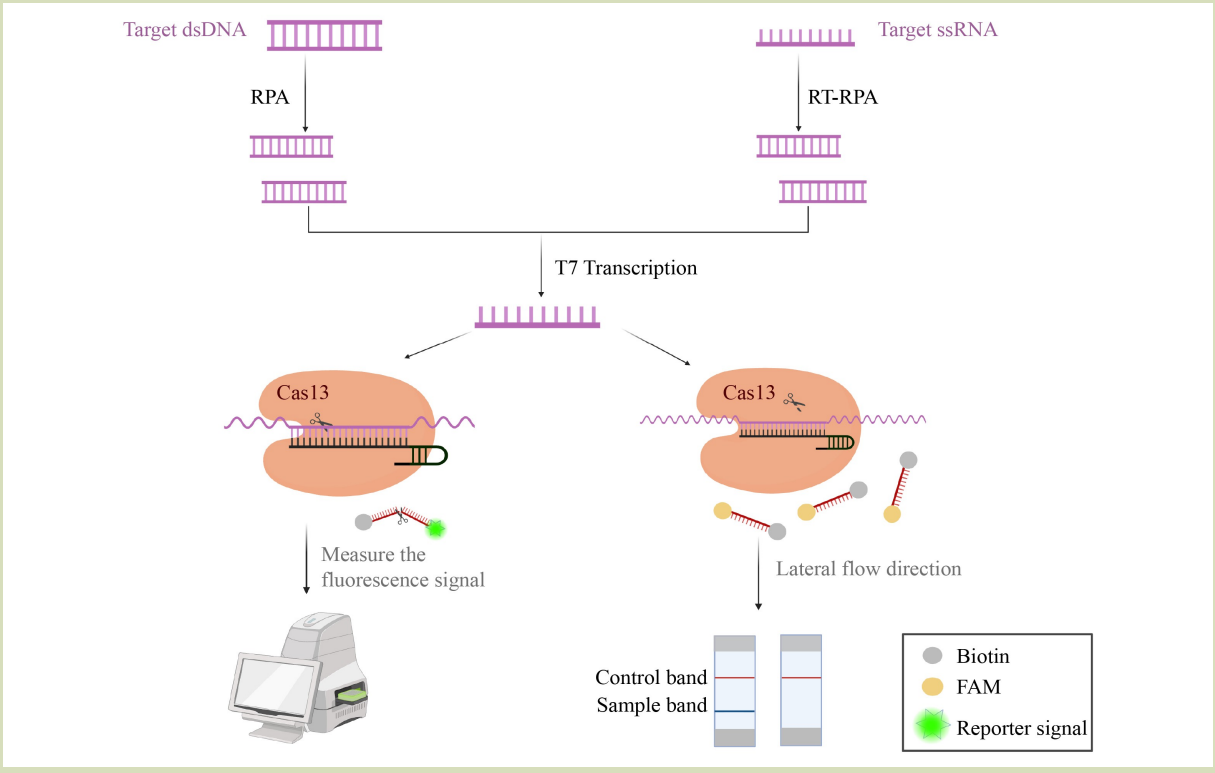


Fig. 7 Schematic of the SHERLOCK Detection System. The SHERLOCK system uses RPA or RT-RPA to amplify target DNA or RNA. The amplified DNA is transcribed into RNA through T7 transcription. Cas13 then specifically recognizes and cleaves this RNA, activating its non-specific cleavage activity. This leads to the cutting of fluorescent-quencher ssRNA reporter molecules, indicating the presence of the target nucleic acid. Alternatively, the system can bind to FAM-biotin reporter molecules, allowing the results to be read on a lateral flow strip.

DTMUV E gene using RPA and Cas13a. This method uses lateral flow and fluorescence detection, both capable of single-copy detection. Its performance is comparable to RT-qPCR, making it suitable for use in resource-limited environments. Additionally, the M segment in AIV is relatively conserved. Based on this, Wu et al.[113] developed a universal method for detecting all subtypes of AIV by combining the CRISPR-Cas13a system with RPA technology. To further streamline the procedure and reduce the risk of contamination, researchers used the HUDSON technique to process samples and combined reverse transcription (RT), RPA, and CRISPR-Cas13a collateral cleavage detection into a single step. After recognizing and cleaving the specific RNA target, Cas13a non-specifically cleaved the fluorescently labeled ssRNA probe, generating a fluorescent signal. This released signal could be detected using either a fluorescence reader or a lateral flow strip. However, this optimized single-step method had weaker fluorescence signals compared to the established three-step approach. Further research is required to address this limitation. In addition, AIVs of the H5 subtype rank is among the most common highly pathogenic avian influenza viruses. Li et al.[114] developed RT-RAA primers targeting highly conserved regions of the H5-AIV HA gene and synthesized crRNA sequences accordingly. They established a detection method combining RT-RAA, CRISPR-Cas13a and LFA, which shortened the detection time and simplified the process. Additionally, mixed infections of duck hepatitis A virus type 3 and novel duck reovirus have caused significant losses in the global duck farming industry. Zhang et al.[115] proposed a one-pot RPA-CRISPR Cas12a/Cas13a strategy, referred to as the Dual RPA-CRISPR Fluorescence System, for simultaneous diagnosis targeting the S3 gene of novel duck reovirus and the *VP1* gene of duck hepatitis A virus type 3. In the Dual RPA-CRISPR Fluorescence System, standard primers were designed to amplify the conserved fragment of the *VP1* gene, which was recognized by the Cas12a system, while T7 promoter-tagged

primers were used to amplify the conserved fragment of the *S3* gene for Cas13a detection mediated by T7 transcription. For Cas12a detection, a FAM-TTTTTT-BHQ1 probe was used, and for Cas13a detection, a ROX-UUUUU-BHQ2 probe was used to achieve accurate differentiation of both genes. The trans-cleavage activity of Cas12a/Cas13a was triggered, leading to the cleavage of the DNA/RNA probes and the generation of a fluorescent signal. They also developed a dual RPA-CRISPR combined lateral flow analysis platform, referred to as DRC-LFA. This platform is optimized for on-site detection by replacing the fluorescence probes with a FAM/biotin DNA probe (300 nm) and a Dig/biotin RNA probe. The RPA amplification system is positioned at the bottom of the tube whereas the CRISPR/Cas detection system is located in the cap. Following the RPA amplification, the tube is briefly centrifuged to ensure mixing and minimize aerosol contamination that could occur from opening the cap.

5 Summary and outlook

This review provides a comprehensive overview of recent advances in Class 2 CRISPR-Cas systems, particularly Cas9, Cas12 and Cas13, in the detection of nucleic acids of animal pathogens. Detection platforms are categorized based on effector protein type, accompanied by an in-depth discussion of their integration with isothermal amplification techniques such as RPA, LAMP and EXPAR. In addition, various signal output modalities are presented, including fluorescence, lateral flow strips and electrochemical biosensors. Notably, this review evaluates CRISPR-based detection from the perspective of veterinary diagnostics, in contrast to the majority of the literature that primarily focuses on applications in human medicine. As such, the review not only provides a systematic summary of technological approaches, but also offers a theoretical and methodological foundation to support the translation of molecular biosensing technologies into veterinary practice.

Despite the considerable advantages of CRISPR-Cas diagnostics, their widespread implementation in veterinary clinical contexts remains constrained by multiple intrinsic and extrinsic factors. From a technical standpoint, the dependency on nucleic acid amplification represents a major bottleneck that hinders deployment in the field. Although amplification steps significantly enhance detection sensitivity, they also introduce risks of cross-contamination, increase workflow complexity and raise overall costs, all of which reduce feasibility in resource-limited or field-based environments. To address this, some studies have proposed one-pot reaction systems that integrate amplification and CRISPR recognition in a single reaction vessel, thereby simplifying the operation and reducing contamination risks. Also, amplification-free detection strategies have received growing attention in recent years. Researchers have proposed various approaches to achieve high-sensitivity detection without amplification, such as digital droplet-based single-molecule detection[116,117], SERS-based platforms[118] and smartphone-integrated microscopy systems[119]. While these strategies show considerable promise under laboratory conditions, their performance in complex veterinary samples and real-world settings remains to be thoroughly validated. Meanwhile, current CRISPR research is still heavily concentrated on human disease diagnostics and dedicated adaptation for animal pathogens is relatively lacking. For example, the collateral cleavage activity of Cas13, while highly effective in signal amplification, is prone to non-specific activation in crude animal samples, generating background signals and false positives that significantly reduce detection specificity and reliability.

From a biological perspective, the genetic diversity of animal pathogens and hosts presents significant challenges for gRNA design. Given that CRISPR targeting relies on highly specific base-pairing, sequence polymorphisms between species or among pathogen subtypes could result in target mismatches, false negatives or reduced assay universality. In addition, non-specific cleavage and off-target effects, which are intrinsic to certain CRISPR systems, could further contribute to false-positive results. Endogenous inhibitors commonly found in animal samples, such as hemoglobin, bile salts and proteases, can also interfere with enzyme activity. These biochemical interferences are often difficult to eliminate through standard sample preparation protocols, especially under low-resource conditions, and can significantly impact the reliability of the detection system.

From regulatory and practical perspectives, the lack of unified validation standards for CRISPR-based animal diagnostics remains a critical obstacle to clinical translation. Unlike human molecular diagnostics, which are regulated by established systems such as the Food and Drug Administration and Conformité Européenne - In Vitro Diagnostic, veterinary diagnostics fall under the jurisdiction of regional authorities,

such as the World Organisation for Animal Health, United States Department of Agriculture and European Food Safety Authority, where requirements vary widely. Most of these agencies have yet to define specific evaluation criteria for CRISPR-based assays, such as sensitivity, repeatability, and batch consistency. Also, the cost-effectiveness of deploying CRISPR platforms in decentralized settings like farms or rural veterinary clinics remains uncertain. For small-scale operations with limited equipment and personnel, practical adoption is still hindered by substantial entry barriers.

These limitations are not merely technical challenges, but rather reflect systemic gaps that highlight the need for strategic innovation in molecular design, system integration and translational planning.

Looking ahead, the advancement of CRISPR-Cas diagnostics in the veterinary field will rely not only on improvements in molecular performance but also on application-oriented, multilevel innovations tailored to the complexity of real-world settings. Amplification-free detection platforms, enabled by enzymatic signal cascades, nanomaterial-assisted enhancement or digital droplet arrays, are expected to simplify workflows and reduce contamination risks. In parallel, gRNA design tools based on large-scale pathogen genomic databases and supported by artificial intelligence are anticipated to enhance detection universality and robustness across strains and species. To address interference from complex sample matrices, future detection systems will require high tolerance to inhibitory substances. This could potentially be achieved through engineered Cas enzymes with improved enzymatic properties or through the integration of automated sample processing modules within microfluidic platforms to facilitate seamless sample-to-answer workflows. On the device level, combining lyophilized reagents, smartphone-compatible detection modules and fully automated workflows will be key to enhancing CRISPR usability in resource-poor, decentralized veterinary environments. Importantly, performance metrics such as sensitivity, specificity, false positive/negative rates and time-to-result should be critically benchmarked against existing gold-standard diagnostics, such as PCR and enzyme-linked immunosorbent assay to validate the clinical value of CRISPR-based systems. Also, to be field-viable, these platforms must be evaluated not only for technical accuracy but also for operability, portability, cost-efficiency and robustness under unstable environmental conditions. Ultimately, the sustainable development of CRISPR-Cas technologies in the field of animal health will depend on interdisciplinary collaboration. Such collaboration will be essential to transforming these technologies from conceptual innovations into practical, field-deployable and scalable diagnostic tools for the surveillance of animal infectious diseases.

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Compliance with ethics guidelines

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