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CRISPR-based nucleic acid diagnostics for pathogens

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ABSTRACT

Pathogenic infection remains the primary threat to human health, such as the global COVID-19 pandemic. It is important to develop rapid, sensitive and multiplexed tools for detecting pathogens and their mutated variants, particularly the tailor-made strategies for point-of-care diagnosis allowing for use in resource-constrained settings. The rapidly evolving CRISPR/Cas systems have provided a powerful toolbox for pathogenic diagnostics via nucleic acid tests. In this review, we firstly describe the resultant promising class 2 (single, multidomain effector) and recently explored class 1 (multisubunit effector complexes) CRISPR tools. We present diverse engineering nucleic acid diagnostics based on CRISPR/Cas systems for pathogenic viruses, bacteria and fungi, and highlight the application for detecting viral variants and drug-resistant bacteria enabled by CRISPR-based mutation profiling. Finally, we discuss the challenges involved in on-site diagnostic assays and present emerging CRISPR systems and CRISPR cascade that potentially enable multiplexed and preamplification-free pathogenic diagnostics.

1. Introduction

Pandemic outbreak, particularly coronavirus disease 2019 (COVID-19) epidemic, highlights the demand to enhance surveillance systems and diagnostic capacities for pathogenic biosafety [1–4]. The global COVID-19 pandemic has infected 652 million cumulative cases and caused 6.65 million cumulative deaths, and it still results in nearly 80 thousand new cases per day, as of December 27, 2022 (WHO Coronavirus Dashboard, https://covid19.who.int/). Particularly, mutations in viruses spontaneously occur during their replication, and some of them generally account for the variants of concern (VOCs) that could increase the infection severity and render vaccines ineffective [5]. Besides, mutations in bacteria are often the cause of antimicrobial resistance, and horizontal transfer of resistant gene among bacteria is taken as an important way to confer resistance [6]. Development of the diagnostic tools, especially for point-of-care testing (POCT), capable to infer pathogens and their mutations is highly significant for pandemic control.

Current diagnostic technologies for pathogens mainly dependent on culturing and colony counting, antibody-based assay (such as enzyme-linked immunosorbent assay, ELISA) and nucleic acid tests (such as quantitative polymerase chain reaction, qPCR) [7,8]. Application limits of these diagnostics lie in high-cost, long turnaround time and the dependent on the sophisticated equipment, hindering their use in resource-constrained settings where generally lack diagnostic reagents and testing infrastructure. Nucleic acid-based technologies, that require only knowledge of the pathogen genome sequence enable accurate and early diagnosis. Combining with nucleic acid preamplification endows high-sensitivity, moreover, the improvement of assaying specificity, especially single-mutation resolution, is equally essential for pathogen diagnosis. The clustered regularly interspaced short palindromic repeat (CRISPR)-associated (CRISPR/Cas) system is a promising tool for nucleic acid tests. The evolutionary arms race has led to numerous CRISPR systems with different variations in mechanisms and functionalities. Contributed to the high target sequence specificity and programmability, CRISPR/Cas-based nucleic acid diagnostics have been widely developed for pathogens, and some of which are suitable for on-site detection via integrating with lateral flow papers and mobile phones [9–11].

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In this review, we introduced the evolution of the class 1 and class 2 CRISPR/Cas systems as well as the major advances of their applications in nucleic acid diagnostics (Fig. 1). We then presented the applications of CRISPR/Cas-based nucleic acid techniques in pathogenic diagnostics, mainly including viruses, bacteria and fungi. Particularly, we highlighted the application for detecting viral variants and drug-resistant bacteria enabled by CRISPR-based mutation profiling. The challenges such as the development of preamplification-free assay and the potential of CRISPR cascade and new emerging CRISPR systems have been discussed.

2. CRISPR toolboxes

CRISPR/Cas systems pose important roles in the adaptive immune system, and are widely discovered in about 87% archaea and 47% bacteria [12]. CRISPR/Cas systems function as the highly specific molecular scissors to shear invading nucleic acids. A surveillance CRISPR/Cas scissor primarily depends on a ribonucleoprotein assembled from a Cas effector complex and a single guide RNA (sgRNA) composed of a non-coding CRISPR RNA (crRNA) and a *trans*-activating crRNA (tracrRNA) [13]. According to the difference of Cas genes and their proteins, CRISPR/Cas systems were divided into 2 distinct classes and 6 types [14]. Class 1 systems have multisubunit effector complexes composed of multiple Cas effectors, some of which are responsible for pre-crRNA processing, some for crRNA binding and some for nuclease cleaving, whereas all functions in class 2 systems are executed by only a single Cas effector (Fig. 2). Class 1 system contains type I, III and IV, while in class 2 system there also have three types: type II, V and VI. Several promising CRISPR/Cas variants mainly in class 1 system have been engineered, including a series of dead Cas effector and other mutants represented by Cas9 (H840A) and Cas9 (D10A) [15].

2.1. Class 1 CRISPR toolbox

A typical feature in class 1 system that distinguishes from class 2 is the assembly of multisubunit effector complexes (the CRISPR-associated complex for antiviral defence). The mutisubunit effector module consists of Cas3, Cas5-Cas7, Cas8, Cas10 and Cas11, in different class 1 combinations, the effector module contains different Cas proteins relying on the types and subtypes (Fig. 2). Additionally, upstream pre-crRNA processing in class 1 system is directly catalyzed by Cas6, whereas in subtype I−B/C systems, Cas5 seems to functionally replace Cas6 [16]. All the class 1 subtypes by far have been employed in developing nucleic acid diagnostic technologies except for type IV, therefore, we next focus on the type I and III.

Class 1 type I system is further subdivided into 7 subtypes (I-A to I-G) [14]. The signature *Cas3* gene (or variant *Cas3'*) existed in the whole of type I loci. *Cas3* gene encodes a helicase-nuclease fusion enzyme: the helicase domain with a demonstrated capacity to dissociate double-stranded (ds) nucleic acids (dsDNA or RNA-DNA duplexes) could be stimulated by a single-stranded DNA (ssDNA) [17,18]; another fused N-terminal HD nuclease domain is involved

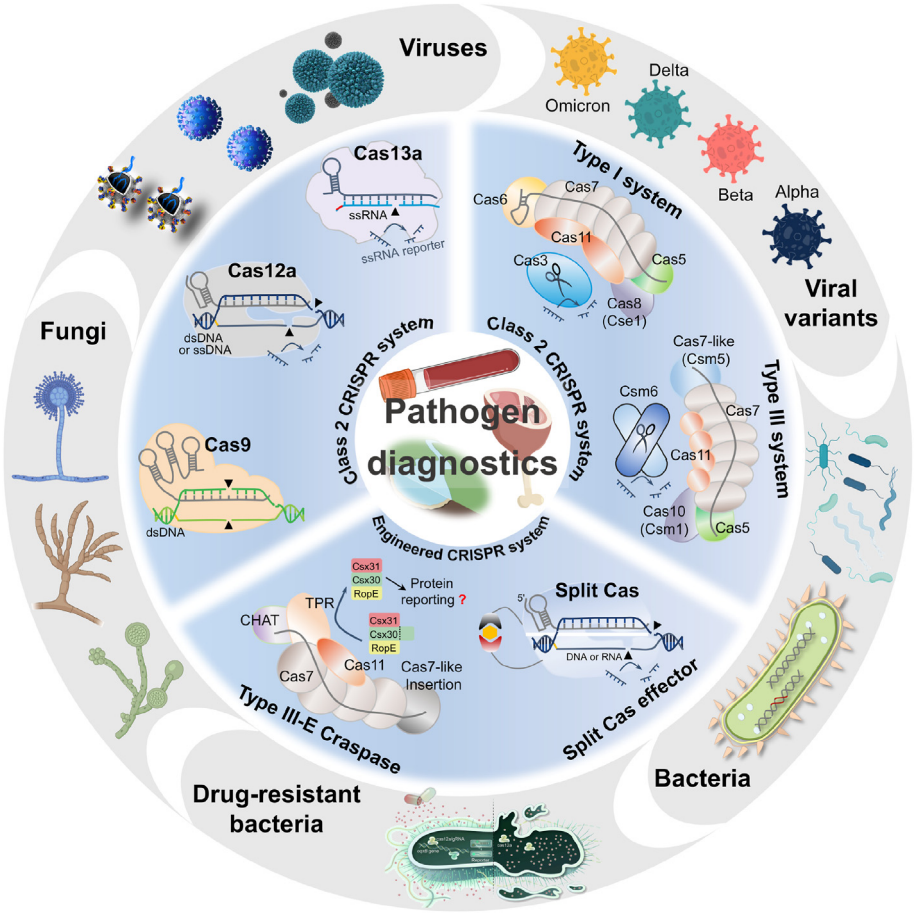

Fig. 1. Overview of CRISPR toolboxes and their applications in nucleic acid diagnostics for pathogens. Class 1 and class 2 CRISPR/Cas system, and engineered CRISPR — Craspase and split Cas effector are designed for detecting viruses, bacteria, fungi, viral variants and mutated drug-resistant pathogens.

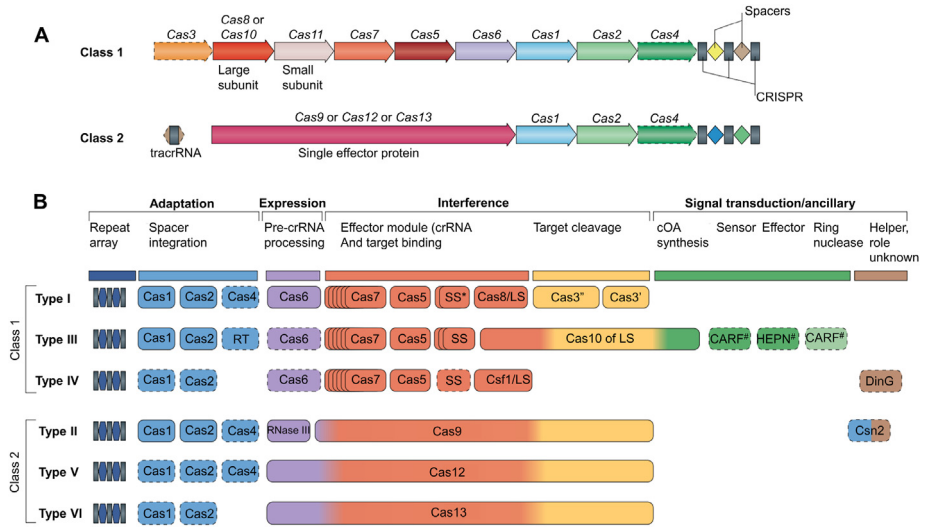

Fig. 2. Classification of CRISPR/Cas system. (A) The overall architectures of class 1 multisubunit effector complex systems that contain type I, III and IV and class 2 single, multidomain effector systems that contain type II, V, and VI [14]. Arrows representing the homologous genes are drawn in the same color. (B) The principal modules of CRISPR/Cas types [14]. The asterisk (*) represents the putative small subunit (SS) that might be fused to the large subunit. The pound sign (#) indicates other unknown sensing domains that might be involved in the signal transduction. Dashed outline indicates the dispensable genes.

in the nick of the non-target strand (NTS) DNA and the processive degradation of the dsDNA near the PAM, and the further robust collateral cleavage upon ssDNA [19]. Type 1 system typically functions to degrade DNA in a two-step process: (i) cascade-mediated identification of the DNA target complementary to crRNA; (ii) target binding-triggered DNA degradation in *trans* caused by the recruited Cas3 [20]. Therefore, the signal reporting of nucleic acid diagnostics using type I systems generally depends on the cleavage activity of Cas3 effector.

Two recent works have developed nucleic acid diagnostics using type I system. The well-defined subtype I-E system was utilized to develop a cost-efficient and equipment-free nucleic acid sensing assay, named Cas3-Operated Nucleic Acid detection (CONAN) [21]. Coupling loop-mediated isothermal amplification (LAMP) pre-amplification with multiple turnover collateral cleavage of ssDNA signal probes by activated Cas3 endows the CONAN assay with a high-sensitivity. It is recently proved that Cas3 and subtype I-A Cascade assemble into an integral effector complex, distinct from the *trans*-recruitment of Cas3 in other type I systems [20]. And preamplification-free nucleic acid detection can be accomplished using this subtype I-A system, and it enabled a pM-sensitivity for dsDNA or ssDNA target within 15 min, while targeting ssRNA yielded a 100-fold lower sensitivity. By contrast, RNA/DNA heteroduplex target requiring at micromolar range could be detected. The use of the lateral strip assay in both work has no trade-off in sensitivity.

Class 1 type III system targets RNAs and consists of subtypes A to E [22,23]. Subtype III-A through III-D systems encompasses the signature gene *Cas10* — also known as Csm1 in subtype III-A/D and Cmr2 in subtype III-B/C systems [24,25]. Cas10 protein, the largest subunit of type III effectors, harbors multiple domains (a HD domain responsible for collateral DNase, two Palm domains for nucleic acid cyclases, and two small α-helical domains) [26]. The multisubunit effector complex of the type III system comprises Cas10 and additional Cas proteins (Csm2-5 in subtype III-A/D or Cmr1 and Cmr3-6 in subtype III-B/C) [27]. Corresponding to Cas3 protein of type I system, Csm6, an auxiliary CRISPR-associated ribonucleases, is responsible for the signal reporting in type III system. Different from the physical association Cas3 with Cascade, the linking of Cas10-containing effector for invader sensing to Csm6 ribonucleases depends on the Cas10-catalyzed cyclic riboadenosines which can act as the activator for Csm6 [28]. Target RNA binding subtype III-A system shows four enzymatic activities: (i) specific *cis*-cleavage upon target RNA by Csm3; (ii) collateral DNA degradation by Cas10; (iii) synthesis of cyclic riboadenosines (cOA) by Cas10; (iv) collateral RNA cutting *in trans* by the cOA-activated Csm6. In subtype III-B system, Cas10 (Cmr2) subunit lacks the HD nuclease domain, and hence lacks the DNase activity [29]. The recently identified subtype III-E is an atypical type III system. Different from other type III system, subtype III-E encodes a single protein (known as gRAMP and Cas7-11) with several domains including one Cas11-like domain, one big insertion domain and four Cas7-like domains (Cas7.1-Cas7.4) [30,31]. Cas7-11 effector cleaves the target RNA complementary to the crRNA at two defined positions. And similar to Cas12 and Cas13, Cas7-11 possesses pre-crRNA processing activity without the need for additional enzymes. Notably, it has been identified that Cas7-11 can associate with TPR-CHAT protease Csx29 (a caspase-like protein) to assemble into Craspase (CRISPR-guided caspase) that capable of RNA-activated cleavage of Csx30 [32–34]. The RNA-activated protein cleavage using Craspase can be explored to sense RNAs either *in vitro* or inside cells [34]. In particular, a CRISPR protease (CalpL) [35] and a NADase (TIR-SAVED) [36] that both can be activated by cyclic nucleotides (cA_4 or cA_3) are recently identified. Combining the proteinase and NADase effectors with CRISPR type III systems holds great potential to develop sensitive assays for RNA sensing.

Several nucleic acid sensing strategies have been reported by utilizing different auxiliary CRISPR-associated enzymes, all of which can be activated by cOA with ring sizes ranging from cA_3 to cA_6, such as Csm6, Can1, Can2 and NucC [28,37–41]. A particular subtype III-A system present in *Lactobacillus delbrueckii* subsp. *bulgaricusthe*, termed LdCsm, is capable of ssDNA cleavage while lacking the cOA signaling pathway [40]. LdCsm enabled sensitive RNA detection at the concentration of 10 nM targets and a single-nucleotide resolution based on the only ssDNase. Sridhara et al. developed a MORIARTY assay (Multi-pronged, One-pot, target RNA-Induced, Augmentable, Rapid, Test sYstem) utilizing both the target-triggered ssDNase and cOA signaling pathways [39]. Multiplex signaling conferred the MORIARTY assay with a higher sensitivity than that using single signaling pathway. In addition, three

detectable Cas10-mediated polymerase products enabled to turn out multiple readouts [38], in which, cOA signaling, pH change during cyclic nucleotide polymerization and pyrophosphate sequestrating the metallic ions allowed for fluorometric and colorimetric detection, respectively. Nucleic acid diagnostics using subtype III-B systems were alike to subtype III-A, mainly performing the cOA signaling that generally using TTHB144 [42] and NucC [37]. Furthermore, subtype III-A system from *Thermus thermophilus* (TtCsm) can simultaneously generate cA_3 and cA_4 that can be respectively recognized by NucC and Can2, thus combining with two ancillary nucleases could improve the sensitivity of nucleic acid diagnostic [41].

2.2. Class 2 CRISPR toolbox

Class 2 systems comprise a single large Cas effector protein — Cas9 (type II), Cas12 (type V) and Cas13 (type VI) — with multidomain that responsible for pre-crRNA processing, crRNA binding and nucleic acid cleavage (Fig. 2) [14]. It is the assembly of multiple domains into a single protein that renders them easily useable. Different from Cas6-executing processing in class 1 systems, pre-crRNA processing in type II systems (Cas9) is carried out by the bacterial RNase III — not a Cas protein. Contrastingly in the bulk type V and VI systems, the distinct catalytic domain responsible for processing is naturally fused in the large Cas effector protein (Cas12 and Cas13).

The first use of engineering CRISPR/Cas9 system for gene editing in eukaryotic cells opens a brand-new era in biotechnology [43]. In type II effector (Cas9), two endonuclease domains, HNH and RuvC, are responsible for nicking the TS and NTS, respectively, upon target DNA binding with the guide RNA (gRNA) [44]. The recognition (REC) lobe of Cas9 protein mediates the recruitment of protein by gRNA, and the protospacer adjacent motif (PAM)-interacting region recognizes the PAM sequences that are beneficial for the preliminary screening of target sequences [45]. Activated Cas9 nicks target dsDNA precisely at 3-nt upstream (5'-side) of the PAM, generating in a blunt-end break. The highly conservative PAM (*e.g.*, 5'-NGG for *Streptococcus pyogenes* Cas9, SpCas9) offers a further improvement of assay specificity for the development of diagnostic tools, however, it also greatly limits the target site selection. To overcome this restriction, a SpCas9 variant, named SpGY, with expanded set of NYN PAMs which is a near-PAMless [46], significantly broadens the applications for CRISPR/Cas tools in nucleic acid tests. Additionally, high demand for diagnostics and gene editing has proliferated a dramatic expansion of class 2 system, including diverse variants. Commonly used Cas9 variants contain Cas9(H840A) that only nicks NTS, Cas9(D10A) that only nicks TS and dead Cas9 that is nuclease-deficient [15]. Cas9(H840A) or Cas9(D10A) can act as a DNA nickase, allowing for the development of isothermal nucleic acid amplification strategies [47,48].

Type V effector (Cas12, previously known as Cpf1), another DNA targeting system in class 2, possesses a collateral cleavage activity on ssDNA in addition to target dsDNA cleavage in *cis*, and the *cis*-cleavage produces a sticky-end break [49]. Cas12a is the first discovered member in Cas12 family, all enzymes of which share similar structural components. Differently, Cas12a only requires a crRNA for guiding target recognition [50], while more compact Cas12 proteins, such as Cas12b and Cas12f (also known as Cas14a) [51,52], need both crRNA and tracrRNA. The PAM for Cas12a commonly including *Lachnospiraceae bacterium* Cas12a (LbCas12a) and its *Acidaminococcus* ortholog (AsCas12a) is 5'-TTTN [50,53], and for *Bacillus hisashii* Cas12b (BhCas12b) is 5'-ATTN [51]. Cleavage of nearby nontarget ssDNA makes Cas12 a promising signal-amplifying element in nucleic acid diagnostic machinery. In addition, Cas12 can target ssDNA and activate the subsequent collateral cleavage activity with no need for PAM [54,55], further promoting the CRISPR/Cas toolbox for nucleic acid tests.

Cas13 family (type VI effectors) is the only one RNA-targeting system in class 2, functions as a ribonuclease [56]. Until now, 6 subtypes have been identified: Cas13a (formerly C2c2), Cas13b, Cas13c, Cas13d, Cas13X and Cas13Y [14], as well as 5 novel subtypes have been deduced based on metagenomic analysis: Cas13e, Cas13f, Cas13g, Cas13h and Cas13i [57]. Cas13 effector adopts a bilobate structure: the REC lobe acts as the binding site for crRNA, and the NUC lobe composed of two higher eukaryote/prokaryote nucleotide-binding (HEPN) domain plays the RNase activity that catalyzes the cleavage of both target ssRNA and bystander nontarget ssRNA. Upon target RNA loading, Cas13 effector undergoes a conformational shift in the NUC lobe to form a stable RNase pocket with the two HEPN domains, which mediate the destruction of invading RNA. RNA-targeting by Cas13 effectors is restricted to the vicinity of a protospacer flanking sequence (PFS).

Nucleic acid diagnostics using the class 2 system have been substantially developed. Cas9 only has the *cis*-cleavage activity that enables no signal amplification, hence generally is coupled with nucleic acid amplification strategies, such as polymerase chain reaction (PCR), rolling circle amplification (RCA) [58], strand displacement amplification (SDA) [59] and LAMP. A Cas9-triggered hairpin probe-mediated biosensing method (termed CHP) for detection of DNA targets was demonstrated utilizing Cas9 as the target recognition element, and RCA coupled SDA as the signal amplification element [59]. This CHP assay achieved a aM-sensitivity and a single-nucleotide resolution, the high-sensitivity and specificity conferred the reliable detection of DNA and its mutations without DNA isolation steps in serum samples. Oppositely, the assay using ligation combined with RCA as target recognition and amplification element and Cas9 as the reporting element was also developed, and it achieved a fM-sensitive for microRNA detection [58]. Cas12 and Cas13 effectors both possess multiple turnover *trans*-cleavage activity that can intrinsically realize signal amplification, potentially enabling preamplification-free detection of nucleic acids [60–62]. Targeting multiplex sites can increase the activation of Cas12 or Cas13 effectors, benefitting sensitivity improvement [63,64]. Nuclease-dead engineering of Cas effectors coverts them from an RNA-guided nucleases to simple binding proteins. dCas proteins assembled with crRNA provide complementary tools for fluorescence *in situ* hybridization (FISH) using nucleic acid-binding proteins, enriching the toolbox for *in situ* single-cell analysis [65].

2.3. CRISPR cascade using class 1 and 2 systems

Attributing to the diverse and abundant CRISPR nucleases, CRISPR cascade strategies were reported in succession [66–70]. Tandem CRISPR nucleases pose the potential for preamplification-free detection with a fM-sensitivity and the improvement of assaying specificity by multiple recognition. The first CRISPR cascade strategy using Cas12a/Cas12f and Cas13a was constructed for nucleic acid diagnostic [66]. The Cas12f-Cas13a cascade assay showed a 16-fold higher sensitivity (~6.25 fM) than Cas12a-Cas13a cascade one for microRNA. The CRISPR/hybrid Cas system that fuses Cas12a and Cas13a to a single protein capable of simultaneously detecting DNA and RNA in a single tube [71]. Another CRISPR cascade strategy for preamplification-free detection of RNA utilized two CRISPR nucleases, Cas13a and Csm6 [67,68]. The ribooligonucleotide A_4-U_6 could be specifically cleaved by target-activated Cas13a, generating A_4>p with a 2',3'-cyclic phosphate that is capable of activating the following Csm6 reporting. Additionally, with the assisted of multiplex PCR or recombinase polymerase amplification (RPA), simultaneously employing multiple Cas

effectors with different signaling pathways, such as DNA-targeting Cas12a and RNA-targeting Cas13a, can establish CRISPR cascade capable of multiplex nucleic acid diagnostics. Two CRISPR cascade platforms have been reported for the authentication of genetically modified (GM) crops via simultaneous detection of featured genes — CaMV35S and T-nos [70], and dual-gene detection of viruses [69].

3. Pathogenic diagnostics

Pathogens, mainly including viruses, bacteria and fungi severely threaten human health [72]. They may have high infectivity and/or toxicity in low doses, For example, the infectious dose of *E. coli* O157:H7 is lower than 100 cells [73]. Notably, genetic mutations in pathogens can potentially lead to an increase of infection risk and severity, and drug-resistance. Besides, a specific pathogenic variant may contain a combination of several mutations, such as the emergent SARS-CoV-2 VOCs —Delta, Omicron [74], and quinolone-resistant *Salmonella* [75]. CRISPR systems have evolved nucleic acid tests on the aspects of sensitivity, nucleotide-resolution, multiplexing and POCT feasibility [76]. We will highlight current diagnostic technologies using the CRISPR toolbox for pathogenic viruses, bacteria, fungi, and their variants or mutations, as well as their contributions to pathogenic safety control.

3.1. Viral infection diagnosis

Viruses are small, infectious, non-cellular pathogens composed of DNAs or RNAs that carry genetic information and an encapsulating protein shell, which can infect human, animals and plants [77]. Nowadays, the outbreak caused by viruses — *e.g.*, Zika virus (ZIKV), African swine fever virus (ASFV), Ebola virus and SARS-CoV-2 — is a persistent threat to public health and global security [78–80]. The mostly recently viral epidemic outbreak is the COVID-19 caused by pathogenic SARS-CoV-2. In addition to person-to-person transmission, SARS-CoV-2 enables person-to-material/material-to-person transmissions [81], highlighting the demand for rapid and low-cost diagnostic methods. Therefore, CRISPR/Cas-based nucleic acid diagnostics have frequently reported for SARS-CoV-2, and other reported targeting viruses include ASFV [64,82], ZIKV [9,79,83], Dengue virus (DENV) [9], Ebola virus [80] and hepatitis B virus (HBV) [84].

The integration of CRISRP with nucleic acid preamplification, such as RPA, PCR and LAMP, enabled sensitive nucleic acid diagnostics. Cas9 and its variants incorporated with preamplification strategies allowed the sensitive detection of viral infection. To achieve low-cost and rapid detection of ZIKV, the reported nucleic acid sequence-based amplification-CRISPR cleavage assay (NASBACC) employed Cas9 to mediate the isothermal NASBA amplification and toehold switch activation that was capable of colorimetric sensing of ZIKV (Fig. 3A) [83]. Additionally, distinct features of Cas12 — multiple turnover *trans*-cleavage, capable of targeting both dsDNA and ssDNA and no requirement of tracrRNA — make it emerging as an alternative for Cas9. Doudna group pioneered the development of a rapid Cas12-based viral diagnostic assay named DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) for human papillomavirus (HPV) diagnosis that yielded an attomolar sensitivity (Fig. 3B) [85]. Sensitivity of the DETECTR assay for viral RNA sensing is suffered by the slow initiation of RPA resulted from the remaining hybridization of complementary DNA (cDNA) to RNA after reverse transcription (RT). Le group took advantage of RNase H to timely remove RNA transcripts from the cDNA-RNA hybrids and release cDNA as the template for RPA, favoring the enhancement of the overall reaction rate of DETECTR [86]. The assay can detect 200 copies S gene of the SARS-CoV-2 RNA within 5–30 min. DETECTR has been widely applied in other virus diagnosis, including SARS-CoV-2 [87], influenza A and B [88], potexvirus and poteyvirus [89]. Apart from RPA preamplification, assays integrating PCR [4] and LAMP [3,90] into DETECTR were capable of detecting 5 copies of SARS-CoV-2. Additionally, the viral diagnostic assay replacing Cas12a in DETECTR with RNA-targeting protein Cas13a, named specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) (Fig. 3C) [9]. SHERLOCK could determine ssRNA of DENV and ZIKV at femto – (~50 fM) and attomolar (2 aM) sensitivity, respectively. Compared with amplification strategies for genomic DNA, NASBA, as the unique RNA amplification strategy is more convenient in assisting RNA-targeting Cas13a assay. Wang et al. reported a Cas13a-based NASBA assay for label-free detection of SARS-CoV-2 aided by a light-up RNA aptamer, yielding a limit of detection (LOD) of 0.216 fM of SARS-CoV-2 pseudoviruses [91].

The emerging class 1 CRISPR systems have been explored for nucleic acid assays for pathogenic viruses. The type I CRISPR system was utilized to develop a cost-efficient and equipment-free nucleic acid sensing assay (CONAN) for SARS-CoV-2 detection (Fig. 4A) [21]. The optimal type of PAM, LAMP preamplification and multiple turnover collateral cleavage of ssDNA signal probes by activated Cas3 endowed the CONAN assay with a LOD as low as 10^2 copies. The foregoing mentioned MORIARTY assay using class 1 type III CRISPR system allowed to detect 2000 copies/μL SARS-CoV-2 in amplification-free settings, attributing to the simultaneous use of *cis*-ssDNase and cOA signaling pathways (Fig. 4B) [39]. And coupling with RT-RPA preamplification, MORIARTY assay significantly improved the sensitivity to achieve a LOD of 60 copies/μL of SARS-CoV-2. Besides, harnessing the changes in pH and pyrophosphate concentration that occur during cyclic nucleic polymerization could realize the colorimetric or visible fluorometric detection of SARS-CoV-2 [38]. Inspired by the use of activator variant that cannot be degraded for TtCsm6 [67,92], Wiedenheft group adopted the Can2 nuclease to assist the signaling of subtype III-A CRISPR capture technique [41]. Use of Can2 lacking the ring nuclease activity can sustain a high level of cA_4 activator, contributing to an sensitivity improvement. Without nucleic acid extraction process, this assay yielded a LOD of 90 fM (5×10^4 copies/μL) for SARS-CoV-2 RNA from nasopharyngeal swab samples.

Separate nucleic acid procedures including preamplification and amplicon detection in viral diagnostics, increase the aerosol contamination risk of amplicon. Uracil-DNA-glycosylase (UDG) can clean deoxyuracil bases existed in amplicons while does not act on natural DNA. In a built UCLD assay (Fig. 5A), UDG clearance occurred before the next round of nucleic acid amplification, which can effectively reduce false-positive diagnosis [93]. Using highly specific primers in the UCLD assay can achieve 100-fold higher sensitivity for SARS-CoV-2 diagnostic than the traditional LAMP assay. Besides, one-tube reaction can alleviate the contamination risk. However, DETECTR or SHERLOCK testing in one-pot generally has trade-off in sensitivity, due to the exponential interval with affection by activated Cas12a and the unavailing consumption of Cas12a activity in the early stages of preamplification. Alternatively, to render the nucleic acid amplification and the Cas protein activation compatible, Chen et al. adopted a photoactivated crRNA to sequentially activate RPA preamplification and Cas12a reporting reaction for ASFV diagnosis (Fig. 5B), favoring that the both processes did not interfere with each other [94]. After RPA preamplification, photolysis under UV lamp of 365 nm degraded photocleavable ssDNA complementary to crRNA, activating the Cas12a effector. This photocontrolled Cas12a strategy could detect as low as 2.5 copies ASFV within 40 min. Moreover, physical separation is also effective in the alleviation of aerosol contamination. Using a disposable tube-in-tube vessel can completely implement

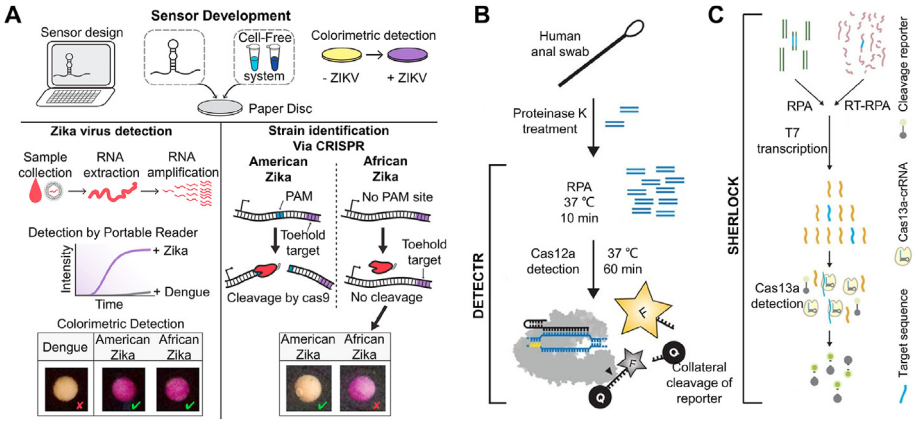

Fig. 3. Preamplification-based nucleic acid diagnostics for viruses. (A) Cas9-based NASBACC assay for detecting DENV and ZIKV [83]. Employing Cas9 to mediate the isothermal NASBA amplification and toehold switch activation allows the visual detection of DENV and ZIKV. (B) Schematic of Cas12-based DETECTR [85]. HPV viral DNA is amplified by RPA, followed by Cas12a-mediated reporting. (C) Cas13-based SHERLOCK assay for detecting DENV and ZIKV [9]. Target DNA or RNA is amplified by RPA or RT-RPA, then the RPA amplicons yield RNA transcripts via *in vitro* transcription, which can activate Cas13a *trans*-cleavage activity for fluorescent reporting.

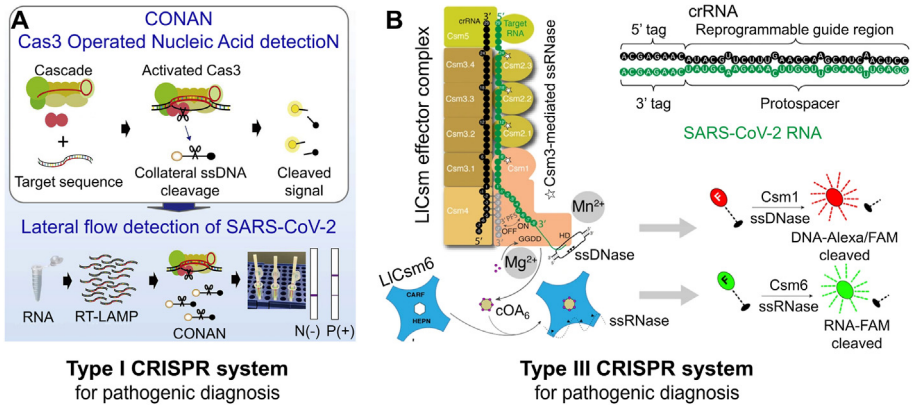

Fig. 4. Class 1 CRISPR/Cas-based nucleic acid diagnostics for pathogens. (A) Schematic representation of CONAN assay using type I CRISPR system [21]. Target-activated cascade can recruit Cas3 effector to achieve collateral ssDNA cleavage, and combining with RT-LAMP enables sensitive detection of SARS-CoV-2 RNA. (B) Schematic representation of MORIARTY assay using type III CRISPR system [39]. LICsm effector complex can be activated by target RNA to exhibit ssDNase and Cyclase activity. Massively produced cOA_6 by Cyclase activity can activate LICsm6 to collaterally cleave ssRNA. Dual signaling by ssDNase and ssRNase activity conferred the MORIARTY assay with an improved sensitivity.

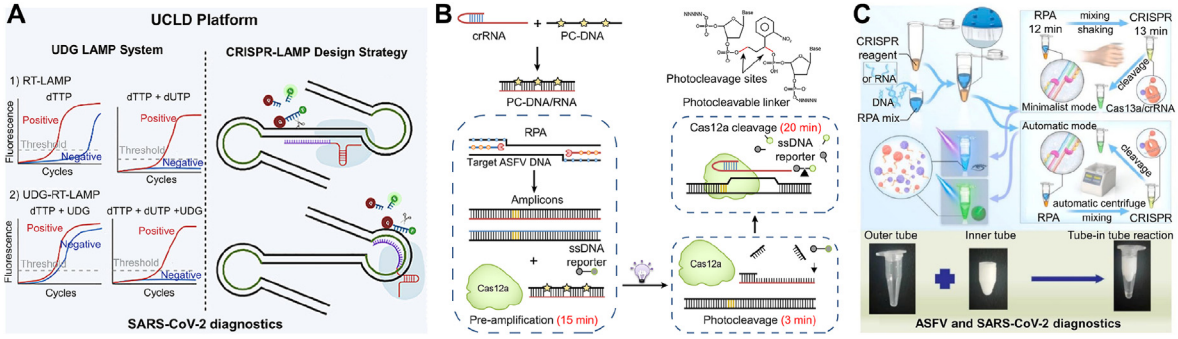

Fig. 5. Contamination-free nucleic acid diagnostics based on preamplification for viruses. (A) Schematic of UCLD-based assay. UDG can clean deoxyuracil bases existed in amplicons while does not act on natural DNA, favoring CRISPR-based LAMP for contamination-free and amplified detection [93]. (B) Schematic of photocontrollable Cas12a-based RPA assay [94]. After RPA preamplification, photolysis under UV lamp of 365 nm degrades photocleavable ssDNA complementary to crRNA, activating the Cas12a effector. (C) Schematic of one-pot Cas13a-based RPA assay using a disposable tube-in-tube vessel to separate RPA preamplification and Cas13a reporting [95].

the physical separation — the inner tube with two hydrophobic holes containing RPA reagent and the outer tube containing pre-stored CRISPR reagents (Fig. 5C) [95]. This strategy was to be verified by testing multiple viruses including DNA virus (ASFV) and RNA virus (SARS-CoV-2), and yielded a LOD of 3 copies/μL.

CRISPR/Cas system with intrinsic signal amplification can serve in preamplification-free nucleic acid detection. Multiplex-crRNA and tandem CRISPR/Cas strategies allow the construction of sensitive nucleic acid diagnostic assay, and have been applied for the diagnosis of DENV, SARS-CoV-2 and Ebola virus. The first work

using multiplex-crRNA strategy combined two crRNAs to enhance the sensitivity of Cas13a assay for SARS-CoV-2 sensing, and the assay achieved a LOD of ~100 copies/μL over 30 min measurement (Fig. 6A) [63]. Another work harnessing multiplex-crRNA strategy in Cas12a system enhanced 64-time sensitivity of the Cas12a-based assay than the conventional single-crRNA system, yielding a LOD of ~1 pM of ASFV genomic DNA (Fig. 6B) [64]. In addition, CRISPR/Cas-only amplification network (CONAN) for HBV DNA detection enabled self/cross catalytic cleavage of nucleic acids, favoring isothermally amplified detection (Fig. 6C) [84]. Target DNA activated Cas12a-crRNA1 complex cleavage the blocking ssDNA in the bubble region, subsequently activating the Cas12a-crRNA2 complex for further positive signal output. The blocking ssDNA partly complementary to crRNA2 was labeled with fluorophore and quencher. The self/cross catalytic amplification empowered an aM-sensitive detection of HBV DNA. Given that the product of Cas13a can activate the following Csm6 cleavage, the tandem Cas13a-Csm6 detection was constructed and exhibited higher sensitivity (20 nM of DENV ssRNA) than that using Cas13a alone [68]. Furthermore, inspired by that blocking the degradation of Csm6 activator may sustain a high level of Csm6 cleavage, Doudna group modified a single 2'-fluoro (2'-F) at the cleavage site of activator to realize the blocking effect. The design of using tandem CRISPR nucleases enabled an amplification-free detection of SARS-CoV-2 RNA (Fig. 6D) [67]. The tandem assay achieved a rapid (<20 min) and one-step detection with a LOD down to 30 copies/μL of SARS-CoV-2 RNA. A DNA walker principle was built as an alternative strategy to amplify Cas13 cleavage reaction. By combining with a DNA walker machine for amplifying the output signal, a preamplification-free Cas13a assay allowed to detect Ebola RNAs with a LOD of 291 aM (~175 copies RNA/μL) [80]. Nevertheless, the design of DNA walker could complicate the nucleic acid assay.

3.2. Viral variant detection

Viral variants may make them competitively fitter in the living environment, and likely bring risks to human health, such as VOCs of SARS-CoV-2 and the 2009 pandemic influenza A virus (H1N1) [96–98]. Omicron variants of SARS-CoV-2 owns mutations, K417 N, T478K, E484A and N501Y that are associated with variations of transmissibility and virulence [97]. The N222 variants of H1N1 frequently occurred in severe and fatal cases [98]. The high specificity of CRISPR-based nucleic acid diagnostics enabled the profiling of viral variants with single-nucleotide resolution. Strategies to ensure CRISPR-based nucleic acid tests to be sensitive to mutations in target genes mainly include the introduction of base mismatches in the guide RNA and the combination with other single-nucleotide polymorphism (SNP)-specific profiling methods (*e.g.*, target-templated ligation and amplification refractory mutation system).

The presence of base mismatch occurring close to PAM region could dramatically reduce the *cis*-cleavage activity of Cas9. Via sgRNA engineering, Cas9 was used to sense mutations in viral RNAs. For example, a CRISPR-FnCas9-based assay mutated the 2nd and 6th positions adjacent to the PAM on the sgRNA enabled to profile mutations in N501Y variant of SARS-CoV-2 (Fig. 7A) [99]. Patient sample testing — mutant type-infected (n = 22) and wild type-infected (n = 37) — showed a 97% accuracy. Combining with lateral flow strips as readouts can realize visual POC diagnostic, Ali et al. employed mutation-containing dCas9/sgRNA complex as a recognition element that specifically bound with FAM-label amplicons (Fig. 7B) [100]. This proposed POC diagnostic allowed sensitive detection of multiple SARS-CoV-2 VOCs — Alpha (deletions of residues 69 and 70), Beta (D215G), Delta (P481R and L452R) and D614G (common to Alpha, Beta, and Delta variants). By incorporating artificial mutations in crRNA, a highly sensitive Cas12a-based assay has been constructed to identify D614G mutant [101]. Utilizing the CRISPR/hybrid Cas system that is capable of sensing of DNA and RNA can achieve simultaneous identification of wild-type SARS-CoV-2 and N501Y variant [71]. Apart from the specificity implemented by Cas effectors, ligation reaction can also be used to discriminate SNPs. Target-templated ligation possesses the capacity of single-mutation discrimination. Wang et al. employed the ligation and transcription to produce the D614G-specific DNA templates that capable of triggering the subsequent transcription. The RNA transcripts can activate the multiple turnover cleavage of Cas13a to further amplify the signal. The adoption of broccoli, a light-up RNA aptamer, as the reporter achieved a label-free detection. This assay yielded a high-sensitivity with a LOD of 82 copies of SARS-CoV-2 (Fig. 7C) [102].

Viral variants are commonly featured with multiple mutations. For diagnostic of Omicron with multiple mutations, Liang et al. refined RT-PCR DETECTR to sensitively sense multiple Omicron SNPs via using engineered two crRNAs that separately covered

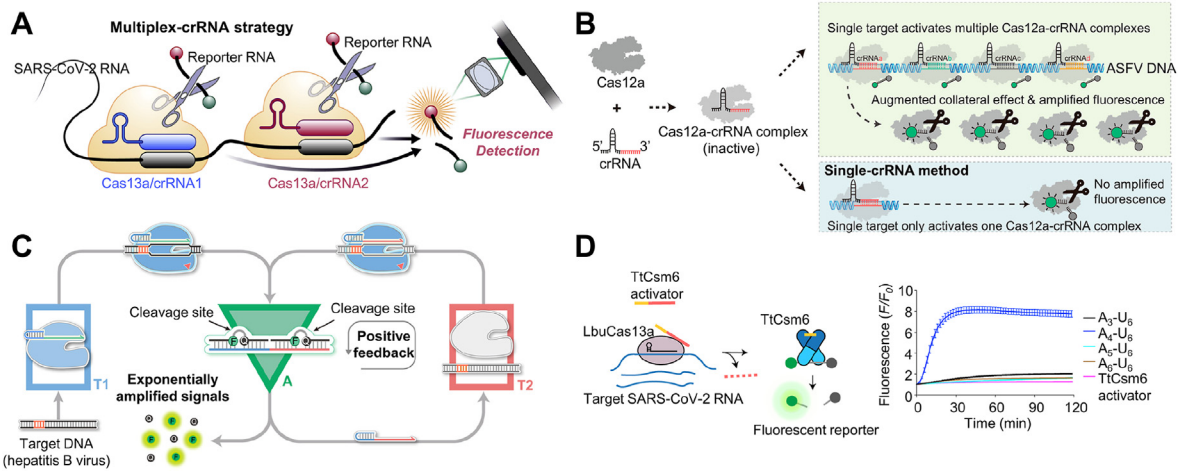

Fig. 6. Preamplification-free nucleic acid diagnostics for viruses. (A) Schematic of multiplex-crRNA strategy using Cas13a for SARS-CoV-2 detection [63]. (B) Schematic of multiplex-crRNA strategy for detecting ASFV [64]. (C) Schematic of CRISPR/Cas-only amplification network (CONAN) for amplified detection of HBV [84]. Target DNA activated Cas12a-crRNA1 complex cleavage the blocking ssDNA at the bubble region, subsequently activating the Cas12a-crRNA2 complex for further positive signal output. (D) Schematic of CRISPR tandem assay using Cas13a and Csm6 [67]. Activated Cas13a can cleavage A_4-U_6 to produce A_4>p that activate the subsequent Csm6 cleavage, achieving cascade signal amplification.

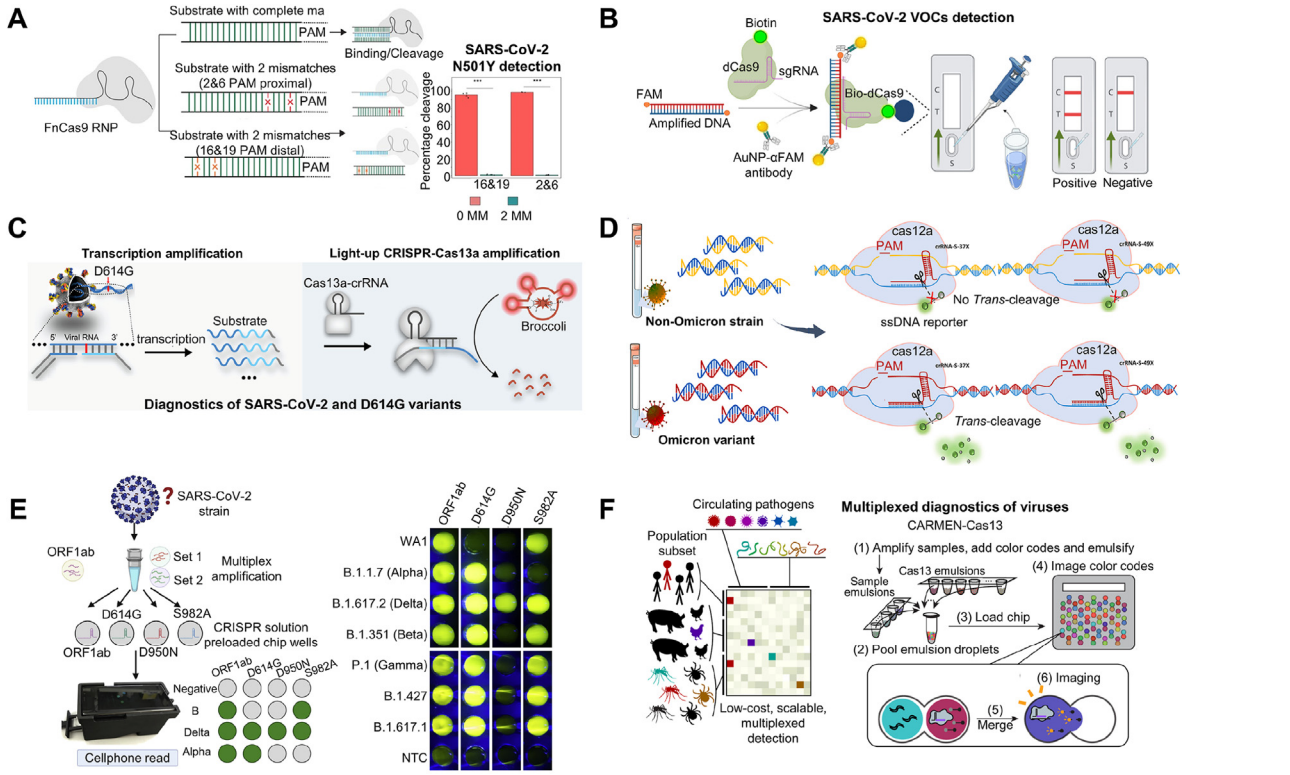

Fig. 7. CRISPR/Cas-based nucleic acid diagnostics for viral variants. (A) Schematic of FnCas9-based assay [99]. Artificial mutating the 2nd and 6th positions adjacent to the PAM on the sgRNA to achieve the single-nucleotide specificity for N501Y variant of SARS-CoV-2. (B) Schematic of dCas9-based lateral flow assay [100]. Using dCas9 as a recognition element that specifically bound FAM-label amplicons and lateral flow strips as readouts, and combining with variant-specific sgRNAs for detecting multiple SARS-CoV-2 VOCs. (C) Schematic of the ligation-triggered Cas13a assay for D614G discrimination. Target-triggered ligation can generate a transcription template with a sequence that is capable of activating the subsequent Cas13a reporting [102]. (D) Schematic of a RT-PCR assay for multiple sensing Omicron SNPs [4]. A crRNA contains multiple specific mutant sites, enabling simultaneous detection of multiple mutations. (E) Schematic of RT-PCR and RT-RPA designed for simultaneous detection of multiple SARS-CoV-2 VOCs [103]. This assay combines multiple RT-PCR and RT-RPA with CRISPR reporting. (F) Schematic of combinatorial arrayed reactions for multiplexed evaluation of nucleic acids (CARMEN) assay for scalable and massively multiplexed detection of pathogenic viruses [106].

three mutant sites (S371L, S373P, S375F for crRNA-S-37 and Q493R, G496S and Q498R for crRNA-S-49X) (Fig. 7D) [4]. The cover of 3–4 mutations evaded the requirement of utilizing multiple crRNAs. A sensitivity of 2 copies per reaction of the Omicron plasmid DNA has been achieved, and this method specifically differentiated Omicron variants from Alpha, Beta and Delta in a test of 57 positive clinical specimens. The combination of multiple RT-PCR and RT-RPA with CRISPR reporting was demonstrated to be able to realize the simultaneous detection of multiple SARS-CoV-2 VOCs (Fig. 7E) [103]. In this assay, the Alpha-and Delta-specific single-nucleotide variants (SNVs) including S982A, D614G and D950 N were able to be identified by PAM motif or seed regions of Cas12a system. Employing a smartphone readout device could detect the SNP-involved SARS-CoV-2 virions with a LOD of 0.5 copies/mL. Thus, this method is promising for high-throughput screening of SARS-CoV-2 infection both in clinical laboratories and POCT settings. In addition, to address the intolerance of Cas proteins to LAMP temperature (55–65 °C), a thermostable Cas12b (BrCas12b) from *Brevibacillus* sp.SYP-B805 was selected, thereby readily enabling one-pot detection [104]. The one-pot BrCas12b-based LAMP assay showed 96.7% accuracy, 99.4% specificity and 92.8% sensitivity in a test of saliva samples for SARS-CoV-2 VOC discrimination including Alpha, Beta, Gamma, Delta and Omicron. Analogous to BrCas12b, selected *Thermoclostridium caenicola* Cas13a (TccCas13a) remains thermal stability at LAMP reaction temperature, even possesses robust cleavage activity at a broader temperature (37–70 °C). This one-pot TccCas13a-based LAMP assay has been demonstrated to detect different SARS-CoV-2 variants [105]. The highest multiplexing assay for pathogenic viruses and their variants was reported by Sabeti group [106]. The assay, termed CARMEN, combined Cas13-based nucleic acid assays with a microwell array which was designed for pairing droplets of amplified samples and sample testing (Fig. 7F), enabling to simultaneously distinguish 169 viruses associated with human health, and multiplex discrimination of dozens of human immunodeficiency virus mutations that are resistant to drug. This assay promises comprehensive testing of scalable samples, however, not applicable for POCT yet due to the involvement of the complexing and costly microwell array.

3.3. Bacterial infection diagnosis

Bacteria cause outbreaks of acute infectious diseases. Combining with preamplification generally confers high-sensitivity to CRISPR/Cas-based diagnostics of bacteria. A PCR preamplification-based Cas12a assay was reported to detect *Vibrio parahaemolyticus* (*V. parahaemolyticus*) with a LOD of 1.02×10^2 copies/μL [107]. Isothermal preamplification can bypass the use of thermal cyclers without compromising the sensitivity. Cas12a-based RPA assay has been used to detect pathogenic bacteria, and enabled a sensitive detection with a LOD of 10^2 CFU/mL for *Salmonella* in egg samples [108], 10 copies of plasmid DNA for *S. aureus*, *E. coli*, and *Listeria monocytogenes* (*L. monocytogenes*) [109], 2 copies/reaction of plasmid DNA for *Brucella* spp. in milk [110] and 2.5×10^3 CFU/mL for *Xanthomonas oryzae* pv. *oryzae* in rice [111]. Using gold nanoparticles (AuNPs) as the reporter of CRISPR assays allowed colorimetric and on-site detection. The AuNPs-based Cas12a assay

empowered the detection of *Salmonella* with a single-cell sensitivity (Fig. 8A) [112]. In addition, integrating with G-quadruplex deoxyribozyme (DNAzyme) that can catalyse 3,3',5,5'-tetramethylbenzidine sulfate (TMB) to oxTMB also allows colorimetric detection. Yin et al. integrated the G-quadruplex with CRISPR to construct a colorimetric assay, and used a smartphone as the signal readout, contributing to a convenient POCT (Fig. 8B) [113]. This Cas12a-based visual assay can detect *Salmonella* as low as 1 CFU/mL.

Unculturable viable but non-culturable bacteria can escape the detection by conventional culture and colony counting methods. DNA-targeting diagnostics fail to identify whether the bacteria are alive because genomic DNA is hard-to-degrade [114]. Oppositely, due to the rapid degradation of RNA in dead cells, RNA-targeting diagnostics can accurately indicate live status of bacteria through timely sensing cellular RNA. NASBA preamplification and Cas13a reporting were incorporated to construct an assay for detecting viable bacteria. Dual signal amplification by NASAB and Cas13a reporting endowed a high-sensitivity with a LOD of 1 CFU and 1% viable *Salmonella* (Fig. 8C) [115].

To evade carryover contamination that often occurs in nucleic acid amplification-based assays, it is feasible to perform one-pot detection. Through the optimization of reaction conditions such as the concentrations of reagents, an one-pot Cas12a-based RPA assay was constructed for detecting *S. aureus* and *E. coli* O157:H7, which yielded a single-cell sensitivity [116]. Alternatively, Cas9 with durable *cis*-cleavage can be applied to degrade undesired amplicons that can lead to cross-contamination. A contamination-

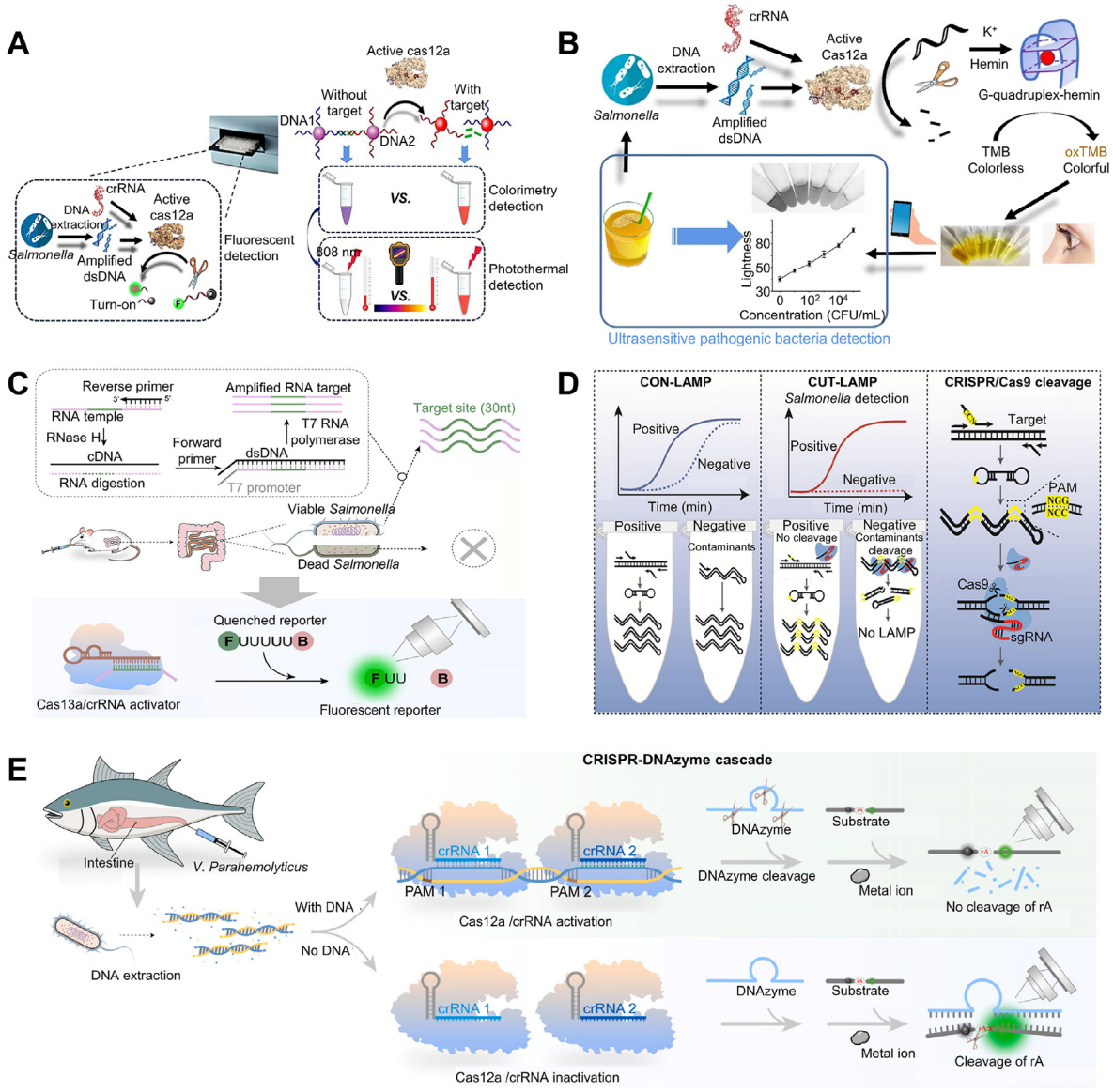

Fig. 8. CRISPR/Cas-based nucleic acid diagnostics for bacteria. (A) Schematic of the AuNPs-based PCR assay for detecting *Salmonella* [112]. Using AuNPs as the reporter of CRISPR assays allows colorimetric visual detection. (B) Schematic of Cas12a- and G-quadruplex-based colorimetric RPA assay for detecting *Salmonella* [113]. G-quadruplex serves as the reporter of Cas12a system, which can produce a turn-off colorimetric signal. And the use of a smartphone as the signal readout can achieve a convenient POCT application. (C) Schematic of the Cas13a-based NASBA assay for detecting viable bacteria [115]. NASBA preamplification produces abundant RNA transcripts that can activate the following Cas13a reporting. RNA sensing can achieve the detection of viable bacteria. (D) Schematic of a CUT-LAMP assay for detecting *Salmonella* [117]. Using Cas9 as the scissor for cleaving undesired amplicons to eliminate cross-contamination. (E) Schematic of Cas12a- and DNAzyme-based cascade assay for the detection of *V. parahaemolyticus* colonized in fish (*perca fluviatilis*) [122]. Target-activated Cas12a can degrade the enzyme strand of DNAzyme, suppressing the DNAzyme reporting.

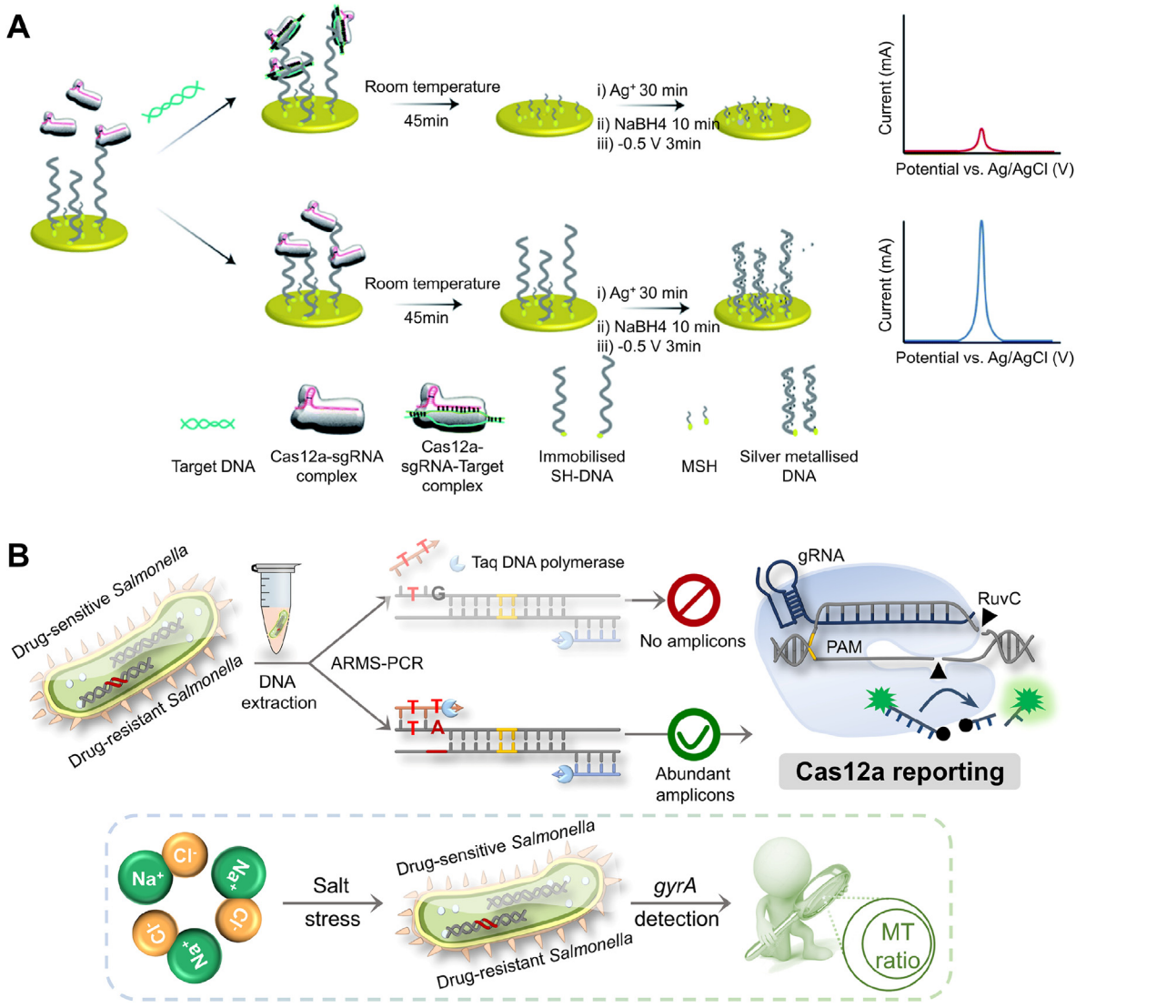

Fig. 9. CRISPR/Cas-based nucleic acid diagnostics for drug-resistant bacteria. (A) Schematic of Cas12a-based electrochemical biosensor for the detection of *mecA* gene of MRSA [125]. (B) Schematic of the cARMS assay for the detection of SNP-involved drug-resistant *Salmonella* [126]. PCR primers designed with artificial mutations improve the capacity for discriminating single-nucleotide variation.

free CUT-LAMP assay adopted the above strategy to alleviate carryover contamination. It yielded negligible false-positive results even with contaminants up to 10 pg while the traditional LAMP assay had a high background in the presence of 1 fg of contaminants (Fig. 8D) [117]. CUT-LAMP reaction can reliably sense *Salmonella* genomic DNA down to 100 copies, and with no background amplification. Cas12a system for the endpoint record of CUT-LAMP products was further tested, it could discriminate the *Salmonella* and *S. aureus* LAMP products. UDG could replace the Cas9 to serve as the cleaner for cross-contaminants. Qian et al. used the UDG degradation to eliminate the cross-contamination [118]. Cas12a can recognize uracil-rich sequences including PAM sequence of 5'-UUUN, enabling the establishment of an UDG-assisted LAMP-Cas12a assay that allowed the alleviation of cross-contamination. This assay has been applied to diagnose infection of *Candidatus* Liberibacter *asiaticus* associated with citrus huanglongbing [119], and yielded a LOD of less than 10 copies.

Combining with functional nucleic acids could achieve pathogenic diagnostics free of preamplification [120,121]. For example, Lu et al. reported an assay via CRISPR in tandem with RNA-cleaving DNAzyme, named CRISPRzyme assay (Fig. 8E) [122]. Dual amplification via multiple-turnover cleavage of Cas12a and DNAzyme enabled preamplification-free detection. The sequential CRISPRzyme assay yielded a LOD of 62 CFU, 86 CFU and 82 CFU for *V. parahaemolyticus*, *Salmonella typhimurium* (*S. typhimurium*) and *L. monocytogenes*. It was further applied to screen antimicrobial probiotics against fish pathogen — *V. parahaemolyticus*, and found that *Bacillus* and lactic acid bacteria isolated from fermented food could serve as potential antimicrobial probiotics. Reduction of background signal would contribute to the improved sensitivity of CRIPSR-based assay. To obtain an ultralow background, the PPCas12a assay designed a pair of DNA reporters labeled with proximal fluorophore and quencher, enabled bacterial detection without preamplification [61]. The proximity of fluorophore to quencher resulted in an ultralow background, favoring that Cas12a directly detect *Salmonella* with a LOD of 619 CFU. The assessment using qPCR indicated that the PPCas12a assay can reliably detect *Salmonella* contamination in complex food matrices.

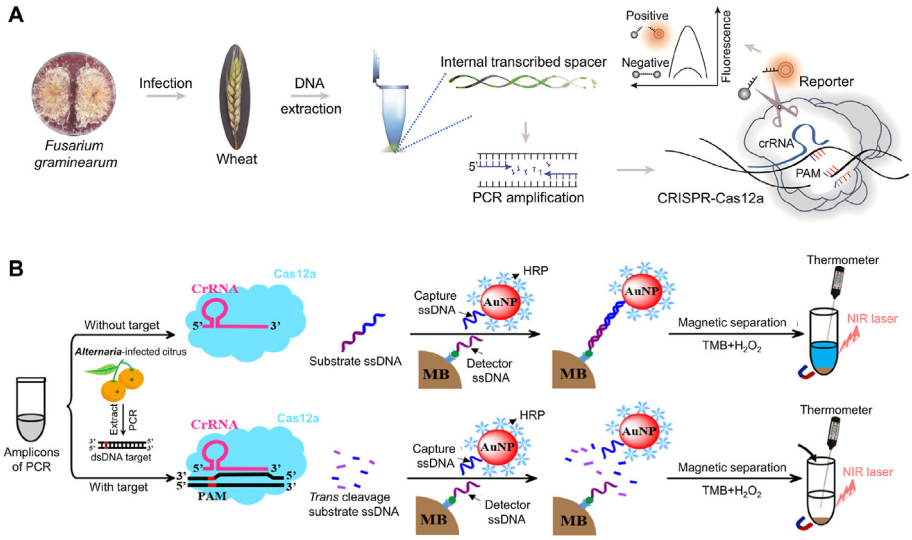

Fig. 10. CRISPR/Cas-based nucleic acid diagnostics for fungi. (A) Cas12a-based PCR assay for the diagnostic of *Fusarium graminearum* infection of wheat [128]. Target DNA was amplified by PCR, followed by Cas12a reporting. (B) Schematic of a Cas12a-based photothermal PCR assay for the colorimetric detection of *Alternaria* genes that encoding the citrus brown spot from citrus [132]. Coupling magnetic separation with the adoption of HRP-loaded AuNPs as the Cas12a reporter can achieve a visual detection.

3.4. Drug-resistant bacteria detection

Overuse of the antibiotics in both the hospital settings and communities has led to the widespread prevalence of drug-resistant bacteria. The resultant antimicrobial resistance in bacteria is often attributed from gene mutations and horizontal transfer of resistant gene among bacteria [6]. Methicillin-resistant *S. aureus* (MRSA) is one of the most important horizontally acquired drug-resistant strain [123]. A FISH-like CRISPR assay using dCas9/sgRNA complex as recognition components was reported for visual detection of MRSA [124]. The 10-fold higher output signal of MRSA than that of methicillin-susceptible *S. aureus* (MSSA) suggested that the FISH-like dCas9 assay was capable of accurate identification of bacterial genotypes. To achieve preamplification-free detection, Suea-Ngam et al. combined sensitive electrochemical readout with silver metallization to construct a Cas12a-based biosensor to detect the *mecA* gene of MRSA (Fig. 9A) [125], yielding a quantification limit of 10 fM and a linearity ranging from 10 fM to 0.1 nM. No trade-off in detection performance was observed when detect MRSA in human serum.

Drug-resistance induced via genetic mutation is challenging to be detected. Cas12a-signaling amplification refractory mutation system (ARMS)-PCR, called cARMS assay was capable of specific differentiation of SNP-involved *Salmonella* via adopting an allele-specific primer with artificial mismatch (Fig. 9B) [126]. The dual-recognition processes — allele-specific primed polymerization and Cas12a/crRNA hybridization — conferred single-nucleotide discrimination capacity. And the dual amplification of PCR and Cas12a reporting rendered the cARMS assay highly sensitive with a LOD of ~0.5% drug-resistant strain that was comparable to qPCR. Isothermal amplification can be used in the CRISPR/Cas-based diagnostics for the identification of SNP-involved bacteria, which would facilitate POCT application.

3.5. Fungal diagnosis

Diseases caused by pathogenic fungi have become a significant issue in both agricultural production and public health security. Increasing nucleic acid diagnostics of fungal infection based on CRISPR/Cas has been developed. The widespread prevalence of wheat scab in wheat-growing period remarkably reduces the wheat yield, and is accompanied by generating varieties of mycotoxins to contaminate food [127]. *Fusarium graminearum* (*F. graminearum*) is the primary causative agent of wheat scab. Integration of Cas12a with PCR enabled the early detection of *F. graminearum* infection (Fig. 10A) [128]. This assay can detect *F. graminearum* with a LOD of 1 fg/μL total DNA that contributed to a successful diagnostic of 4-day *F. graminearum* infection. Furthermore, the diagnosis of invading fungal diseases highlighted the need in developing field-deployable POCT. Assisting with lateral-flow strips to output visual reading, Cas12 integrated RPA assay enabled rapid (<1 h) POC diagnostic of citrus scab with a LOD down to 1 fg crude DNA [129]. To construct a colorimetric readable assay, Liu et al. developed a Cas12a-based photothermal PCR assay by employing AuNPs as a carrier for loading horseradish peroxidase and ssDNA — they are integrated into the Cas12a reporting system (Fig. 10B) [130]. This assay enabled colorimetric nucleic acid diagnostic of *Alternaria* genes that encode the citrus brown spot, and capable of identifying targets from citrus, tomato and apple. Nevertheless, the on-site use of these CRISPR assays is hindered by the need of nucleic acid extraction which can hardly be proceeded in the field. Wei group reported a rapid nucleic acid extraction process using microneedles [131], allowing to obtain pathogenic DNAs within 1 min. The simple sample-pretreatment protocol would facilitate the CRISPR-based nucleic acid tests for in field diagnostics of crop health.

4. Conclusion and outlook

CRISPR-based nucleic acid diagnostics for pathogen infection have been advanced on the aspects of sensitivity, nucleotide-resolution, multiplexing capacity and POCT feasibility (Table 1). However, there are several issues remaining in CRISPR-based tools to be improved. First, a challenge of CRISPR-based nucleic acid diagnostics is the unavoidable use of preamplification for femtomolar targets, which hinders the POCT. Preamplification imposes a burden on assaying simplicity, timeframe and aerosol contamination. Strategies using tandem CRISPR nucleases, and CRISPR with other catalytic molecules (such as enzymes and DNAzymes) have been reported to substitute nucleic acid amplification, and are

Table 1
CRISPR/Cas-based nucleic acid diagnostics for pathogens.

CRISPR tool	Pathogens	Analytes	Preamplification strategy	Assaying time	Sensitivity	Samples	Ref.
Type I CRISPR/Cas3	Virus	SARS-CoV-2	RT-LAMP	30–40 min	10^2 copies	Nasopharyngeal and oropharyngeal swab	[21]
Type III-A CRISPR/Cas assisted with Can2	Virus	SARS-CoV-2	–	35 min	90 fM	Nasopharyngeal swab	[35]
Type III-A CRISPR/Cas assisted with Csm6	Virus	SARS-CoV-2	RT-LAMP	30 min	200 copies/μL	Nasal swab	[38]
Type III-A CRISPR/Cas assisted with Csm6	Virus	SARS-CoV-2	RT-RPA	30 min	60 copies/μL	Nasopharyngeal swab	[39]
Cas13a assisted with Csm6	Virus	SARS-CoV-2	–	20 min	30 copies/μL	Respiratory swab	[67]
Cas9	Virus	ZIKV	NASBA	2–6 h	2.8 fM	Plasma	[83]
Cas9	Virus	SARS-CoV-2 VOCs	RT-PCR	1.5 h	A few copies	Clinical samples	[99]
Cas9	Bacteria	*Salmonella*	LAMP	30 min	100 copies	–	[117]
Cas9	Bacteria	MRSA	PCR	30 min	10 CFU/mL	Cell lysates	[124]
Cas12a	Virus	HPV	RPA	70 min	1 aM	Clinical samples	[85]
Cas12a	Virus	SARS-CoV-2	RT-RPA	5–30 min	200 copies	Respiratory swab	[86]
Cas12a	Virus	SARS-CoV-2 and its VOCs	RT-LAMP	45 min	10 copies/μL (wild-type) and 1% N501Y	Nasopharyngeal swabs	[93]
Cas12a	Virus	ASFV	RPA	40 min	2.5 copies	–	[94]
Cas12a	Virus	ASFV and SARS-CoV-2	RPA or RT-RPA	25 min	3 copies/μL	–	[95]
Cas12a	Virus	ASFV VOCs	–	60 min	1 pM	–	[64]
Cas12a	Virus	SARS-CoV-2 VOCs	RT-RPA	45 min	10 copies	Clinical samples	[101]
Cas12a	Virus	SARS-CoV-2 VOCs	RT-RPA and RT-PCR	95 min or 30 min	0.5 copies/mL	Nasal swab	[103]
Cas12a	Bacteria	*V. parahaemolyticus*	PCR	~100 min	1.02×10^2 copies/μL	Shrimp	[107]
Cas12a	Bacteria	*Salmonella*	RPA	45 min	10^2 CFU/mL	Egg and chicken	[108]
Cas12a	Bacteria	*Salmonella*	RPA	50 min	1 CFU/mL	Beer and juice	[113]
Cas12a	Bacteria	*Candidatus* Liberibacter *asiaticus*	LAMP	45 min	10 copies	Citrus leaves	[118]
Cas12a	Bacteria	*V. parahaemolyticus*	–	70 min	62 CFU	Perch fries	[122]
Cas12a	Bacteria	Drug-resistant *Salmonella*	ARMS-PCR	60 min	~0.5% SNP	–	[126]
Cas12a	Fungus	*F. graminearum*	PCR	58 min	1 fg/μL total DNA	Wheat seedlings	[128]
Cas12a	Fungus	*Alternaria*	PCR	110 min	1.5 pM	Citrus	[132]
Cas12b	Virus	SARS-CoV-2 VOCs	RT-LAMP	10–30 min	12-500 copies/μL	Clinical samples	[104]
Cas12f	Bacteria	*Eberthella typhi* and *Streptococcus pyogenes*	RT-asymmetric PCR	60 min	10^4 CFU/mL	Milk	[133]
Cas13a	Virus	SARS-CoV-2 and its VOCs	NASBA	150 min	0.216 fM	Clinical throat swab	[91]
Cas13a	Virus	SARS-CoV-2	–	30 min	100 copies/μL	Nasal swab	[63]
Cas13a	Virus	SARS-CoV-2	–	20 min	30 copies/μL	Respiratory swab	[67]
Cas13a	Virus	Ebola	–	40 min	175 copies/μL	Serum and blood	[80]
Cas13a	Virus	SARS-CoV-2 and its VOCs	Transcription	180 min	82 copies	Food packages, seafood and throat swabs	[102]
Cas13a	Virus	SARS-CoV-2	RT-LAMP and transcription	80 min	10 copies/μL	Oropharyngeal swab	[105]
Cas13a	Bacteria	Viable *Salmonella*	NASBA	150 min	1 CFU and 1% viable *Salmonella*	Egg shell, beef, pork, duck, chicken, mutton and tap water	[115]

promising to construct preamplification-free assays. Particularly, the recently reported Craspase [32], CalpL [35] and TIR-SAVED [36], the RNA-activated CRISPR protease or NADase that differs from conventional Cas effectors with nuclease function, has the potential for amplified detection via using protein reporters with high catalytic activities. Second, the improvement of assaying specificity capable of single-nucleotide discrimination is critical for diagnosis of pathogenic variants and mutations. Innovation of the crRNA design, such as chemical modification and mutational introduction would be useful to improve the specificity. Additionally, an engineered split Cas12a has the potential for nucleic acid diagnostics [134]. The split Cas12a could be fused with two DNA handles by covalent linkage at two ends respectively [135], and the assembly of split Cas12a depends on the specific binding between the two handles and the target sequence. This target-specific assembly of Cas12a may contribute to the improvement of assaying specificity. Third, the multiplexing assay is demanded for confidently profiling pathogenic infections via screening possible pathogens and their variants. To this end, efforts to implement multiplexing Cas effectors and cooperate with microfluidic devices may be considered. Based on the found of Craspase, different reporting approaches — nucleic acid reporter and protein reporter — could be incorporated. We can further excavate the tools in the class 1 CRISPR system, which may be considered in multiplex nucleic acid diagnostics [136]. Fourth, on-site use of CRISPR-based assays is hindered by the need of nucleic acid extraction which can hardly be proceeded without chemical reagents or instruments. Combining with rapid nucleic acid extraction processes, such as using microneedles [131], would facilitate CRISPR-based nucleic acid tests for in field or on-site diagnostics. Finally, the difficulty in transport and storage of bioreagents such as Cas enzymes and crRNAs makes the tests not feasible for POCT applications. Reagent storage strategies including lyophilization, nanocarriers and hydrogels are promising for bioreagent preservation. Particularly, the emerging so-called “self-contained” microfluidic devices, which can pre-store all necessary reagents in premeasured quantities, have the potential for

constructing mix-and-read and stabilized assays that suitable for POCT [137]. Collectively, enrichment and evolution of CRISPR toolbox, along with their integration with other analytical devices or materials, will facilitate nucleic acid diagnostics and pathogenic biosafety control.

CRediT authorship contribution statement

Hao Yang: Investigation, Writing-Original draft preparation, Table and figure preparation. Yong Zhang: Reference summarization, Formal analysis. Xucong Teng: Investigation, Resources, Formal analysis. Hongwei Hou: Investigation, Formal analysis. Rujiie Deng: Writing- Reviewing and Editing, Conceptualization. Jinghong Li: Writing- Reviewing and Editing, Conceptualization, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

No data was used for the research described in the article.

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