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REVIEW ARTICLE

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CRISPR/Cas system and its application in the diagnosis of animal infectious diseases

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Abstract

Infectious diseases are a serious threat to the existence of animals and humans' life. In the 21st century, the emergence and re-emergence of several zoonotic and non-zoonotic global pandemic diseases of socio-economic importance has affected billions of humans and animals. The need for expensive equipment and laboratories, non-availability of on-site testing abilities, with time-consuming and low sensitivity and specificity issues of currently available diagnostic techniques to identify these pathogenic micro-organisms on a large scale highlighted the need for developing cheap, portable environment friendly diagnostic methods. In recent years, these issues have been addressed by clustered regularly interspaced palindromic repeats (CRISPR)-based diagnostic platforms that have transformed the molecular diagnostic field due to their outstanding ultra-sensitive nucleic acid detecting capabilities. In this study, we highlight the types, potential of different Cas proteins, and amplification systems. We also illuminate the application of currently available CRISPR integrated setups on the diagnosis of infectious diseases, majorly in food-producing animals (pigs, ruminants, poultry, and

Abbreviations: AHPND, Acute hepato-pancreatic necrosis disease; ASFV, African swine fever virus; BVDV, Bovine viral diarrhea virus; CaPV, Capri pox virus; CCT, canine cyclic thrombocytopenia; CME, Canine monocytic ehrlichiosis; CPV-2, Canine parvovirus type 2; CRISPR, Clustered regularly interspaced palindromic repeats; ddPCR, droplet digital PCR; DETECTR, DNA nuclease targeted CRISPR trans-reporter; dRPA, dual recombinant amplification system; DTMUV, Duck tumbusu virus; ECMV, Encephalomyocarditis virus; EXPAR, exponential amplification method; FAdV, Fowl adenovirus; FCV, feline calicivirus; FHV, Feline herpes-1; FL, fluorescence; FMDV, Foot and mouth disease virus; HHPS, hepatitis hydro-pericardium syndrome; HOLMES, one-hour low-cost multipurpose high efficient system; HPAIV, Highly pathogenic avian influenza virus; HPV, Human papilloma virus; IBV, Infectious Bronchitis virus; IHN, Infectious hematopoietic necrosis; JEV, Japanese Encephalitis virus; LAMP, loop mediated isothermal amplification; LFD, lateral flow dipsticks; LFS/D, lateral flow strip/disk; LMBV, Largemouth bass ranavirus; LSDV, Lumpy skin disease virus; MIRA, multi-enzyme recombinant amplification; MTBC, Mycobacterium tuberculosis complex; NASBA, nucleic acid sequence-based amplification; NASBACC, nucleic acid sequence-based amplification CRISPR cleavage; NE, naked eye; NEA, nicking enzyme amplification; NECR/D, naked eye colorimetric readout/detection; PAM, protospacer adjacent motif; PCLV, Porcine circovirus-like virus; PCR, polymerization chain reaction; PCV, Porcine circovirus; PD-Co, Porcine delta-corona-virus; PDNS, Porcine dermatitis and nephropathy syndrome; PEDV, Porcine epidemic diarrhea virus; PFS, protospacer flanking sites; POC, point-of-care; PPV, Porcine parvovirus; PRRSV, Porcine reproductive and respiratory syndrome virus; PRV, Pseudorabies virus; RAA, recombinant aided amplification; RCA, rolling circle amplification method; RPA, recombinant polymerization assay; SADS-Cov, Swine acute diarrhea syndrome coronavirus; SCAR, sequence characterized amplified region; SDA, strand displacement amplification; SERS, surface-enhanced Raman scattering strategy; SHERLOCK, specific high-sensitivity enzymatic reporter unlocking; SIBA, strand invasion-based amplification; SIVD, Swine idiopathic vesicular disease; SVA, Senecavirus A; SVV, seneca valley virus; TGEV, Transmissible gastroenteritis virus; TiLV, Tilapia lake virus; TSV, Taura syndrome virus; VD, visual detection; WSSV, White spot syndrome virus; YHV, Yellow head disease virus; ZIKV, Zika virus.

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aquaculture), domestic pets (dogs and cats), and diseases of zoonotic importance. We conclude the challenges and future perspectives of using these systems to rapidly diagnose and treat other infectious diseases and also develop control strategies to prevent the spread of pathogenic organisms.

KEYWORDS

applications, biosensor, CRISPR/Cas systems, infectious disease, molecular diagnostics, point-of-care

1 | INTRODUCTION

Over the last few years, one can observe the increasing surge in the emergence of various new and old animal and human diseases,[1] like Avian influenza, African swine fever, Swine flu, MERS, COVID-19, Ebola, ZIKA virus, brucellosis, lumpy skin disease, tuberculosis, dengue, toxoplasmosis, and many others.[2] These diseases are caused by a number of pathogenic microorganisms such as viruses, bacteria, parasites, protozoa, and fungi.[3] Infectious diseases are the serious hazards to human and animal health as they account for a large number of deaths globally and cause significant economic losses.[4] Infectious diseases spread across communities within no time, making it essential to promptly identify the causative organism and rapid diagnoses of the affected individuals and animals.[5]

A medicine lacks direction without a diagnosis. In the 21st century, a wide variety of diagnostic tests have been designed[6] including nucleic acid-based detection,[7] pathogen culture,[8] nanotechnology-based methods,[9] and protein-based assays.[10] Among these assays, pathogen culture is laborious, requires a lot of time and needs special expensive experimental equipment and laboratories,[11] for example, biosafety levels 3 and 4 requirement for culturing Brucella, Mycobacterium tuberculosis, Bacillus anthracis, and CCHF, EBOLA, Nipah virus, and so on, respectively.[12] Protein-based assay requires the need of antibodies production against specific antigens. Rapid, cheaper antigen detection with high sensitivity, specificity, and reliability has been achieved using nucleic acid-based sequences.[13] For this method, nucleic acid is isolated from biological samples, followed by detection with polymerization chain reaction (PCR), quantitative, genomic sequencing, or other isothermal amplification methods. But, the need for skilled workers and expensive equipment's limits the use of PCR/qPCR,[14] while isothermal amplification methods are less specific and sensitive.

There is no effective vaccine or treatment available for a number of infectious human and animal diseases.[15] This also emphasized the critical importance of developing robust diagnostic methods with the ideal characteristics of being inexpensive, instrument-free, without the need for experienced laboratory workers, and easily accessible with high specificity and sensitivity to prevent and control the spread of infectious diseases.[5] In 1987, the breakthrough discovery of clustered regularly interspaced palindromic repeats (CRISPR) in *Escherichia coli*,[16] the disclosure of associated proteins, its role in bacterial adaptive immunity[17] to safeguard the cells against harmful genetic invaders like plasmids, viruses, and bacteriophages, and meaningful implementation of the CRISPR system in genome editing,[18] genetic engineering,[19] imaging technology,[20] and molecular detection[21] has further transformed clinical achievements and clinical research.

The ability of Cas proteins to target specific nucleic acid sequences and cleavage of the nearby single-stranded DNA/RNA using sgRNA offers potential for genome-editing and led to the development of CRISPR diagnostic assays. The most widely used Cas 9 protein for genetic engineering and previously commonly used for DNA targeting, do not harbor the collateral cleavage activity.[22] The diagnostic systems, PC REPORTER[23] and CARP,[24] combine Cas9 and PCR to detect *Mycobacterium tuberculosis complex* (*MTBC*) and *human papilloma virus*, respectively. Pardee et al. (2016) manufactured NASBACC, integrated nucleic acid sequence-based amplification and Cas 9 to detect the Zika virus at high sensitivity.[25] Following that, the successful identification of two new Cas proteins, Cas12 and Cas13, significantly impacted the nucleic acid diagnostic field. They possess collateral cleavage activity,[26] and require specific rigid protospacer adjacent motif (PAM) sequences to recognize and target DNA/RNA.[27] This led to the foundation of novel CRISPR diagnostic platforms, DETECTR[28] (DNA nuclease targeted CRISPR trans-reporter), HOLMES[29] (one-hour low-cost multipurpose high efficient system), and SHERLOCK[30] (specific high-sensitivity enzymatic reporter unlocking). These systems were developed to diagnose *HPV*, *Pseudorabies virus* (*PRV*), and *Zika virus* (*ZIKV*), respectively. But nowadays, based on these methods and with relevant modifications a lot of human and animal diagnostic methods have been developed. The newly discovered Cas protein, Cas14,[31] possesses a collateral activity for ssDNA but it

recognizes and cleaves ssDNA without the need for any PAM sequence.[32] Recently, taking advantage of PAM free Cas14 protein, Wei et al. made SPCas, *Salmonella typhi* detection assay.[33]

In the last few years, CRISPR technology has evolved significantly, moving beyond Cas9 to include other Cas proteins (Cas12, Cas13 and Cas14), which provide exceptional sensitivity to detect pathogens.[34] The ability of CRISPR systems to operate at room temperature, elimination of primers for amplification-free assays,[35] freeze-drying of CRISPR reagents for field use, transformation of molecular diagnostics from tube to POC devices (mobile phone apps,[36] glucometer[37]), advancement in microfluidic,[38] and nano-technology[39] and its coupling with CRISPR systems, and above all its successful worldwide application during COVID-19 pandemics,[40] have made CRISPR diagnostic, a hot topic in recent years. Therefore, it is important to summarize how different CRISPR effectors can be coupled with nucleic acid detectors for diagnosing infectious diseases in animals. This review highlighted a short summary of the characteristics of different classes of CRISPR systems. Secondly, we discussed the pros and cons of different amplification systems and classified the CRISPR-based diagnostic platform for diagnosing infectious diseases in different animals. In addition, we shed light on the challenges of the CRISPR system, and possible solutions and convey potential insights for future innovation.

2 | CRISPR/CAS CLASSIFICATION

The CRISPR molecular scissors have been categorized into two sets of primary groups,[41] that feature 6 types and 48 subtypes. The class 1 CRISPR–Cas system can be classed into a trio of groups: types 1, III, and IV and 33 subtypes.[42] Around 90% of CRISPR–Cas systems fall under Class I, majorly found in fungi and a minor percentage in bacteria.[43] These types cut the targeted nucleic acid sequence using interference machinery that comprised of a combination of several Cas effectors and crRNA.[44] The unique feature of the type 1 class is the adoption of multiple Cas protein complexes (4–7 protein subunits) to execute nucleic acid detection. The design and complexities of these systems and also development of several heteromeric RNA-guided Cas nucleases, complicate their integration into diagnostic platforms.[45] In contrast to the Class I system which requires multiple Cas effectors to form a complex with crRNA for the surveillance and cleavage of nucleic acid, the Class II system depends only on a single multi-purpose protein.[46] Due to simple organization and distinct architecture, around 95% of the CRISPR diagnostic platforms were developed using Class II system enzymes.[47,48]

The second system of CRISPR–Cas, majorly present in bacteria, also consists of three main types II, V, and VI, each with unique nucleases Cas9, Cas12/14, and Cas13, respectively. The most well-known Cas protein, Cas9, belongs to type II (four sub-types) of the class II CRISPR/Cas system. This protein for the recruitment and guidance to cleave a specific DNA sequence, requires dual RNA, trRNA, and crRNA. Cas9 protein, mediated by the crRNA–trRNA complex, recognizes the specific PAM[49] region (5′-NGG-3′) on DNA and subsequently its two domains, HNH and RuvC, cleaves dsDNA to produce blunt ends.[50] Cas12 and Cas14 are the two key proteins of the type V system. Cas12 binds with only crRNA to form a complex (crRNA–Cas12) that identifies the specific PAM sequence (5′-(T)TTN-3′) on the targeted DNA.[51] On the contrary, Cas14 (composed of only 700aa), mainly breakdown ssDNA and does not require any specific T-rich PAM sequence.[52] Both these proteins have collateral activity, once coupled with targeted DNA, they can break down any adjacent non-targeted DNA. Type VI system consists of five variant subtypes and cas13 is the distinguishing protein of this system possessing two HEPEN RNAase domains. Likewise type V, does not need any tracrRNA and crRNA–Cas13 identifies the specific protospacer flanking sites (PFS) and cuts the targeted ssRNA.[53] Figure 1 highlights the summary of different Cas proteins and their cleavage activities.

3 | AMPLIFICATION STRATEGIES AND CRISPR SYSTEM

The development of an efficient CRISPR-based biosensor is critically important for pathogen detection in animals and this biosensor detects infectious particles[54] mainly involving five key steps (Figure 2): selection of samples (food, milk, blood, urine, milk, etc.), identification of the targeted DNA or pathogen (bacteria, virus, parasite, etc.), amplification of the nucleic acid (DNA/RNA), selecting an appropriate CRISPR–Cas system (Cas9, Cas12, Cas13, and Cas14), and signal transduction methods[55] (fluorescent, lateral flow strips, Colorimetric readout, and naked eye).

The identification of the targeted pathogen is an important and challenging step in animal-related studies because of the complex sample nature like meat or milk, and due to minute amounts of the targeted analytes in the samples. It is important to critically select the amplification method as PCR-based amplification needs a lot of time and requires complex instruments.[56] Similarly, recombinant polymerization assay[57] is simple, rapid, and operates at 30–40°C, but counter aerosol contamination, off-target nucleic acid contamination, and variable sensitivity and specificity issues. The multi-enzyme recombinant amplification[58] (MIRA) is widely used due

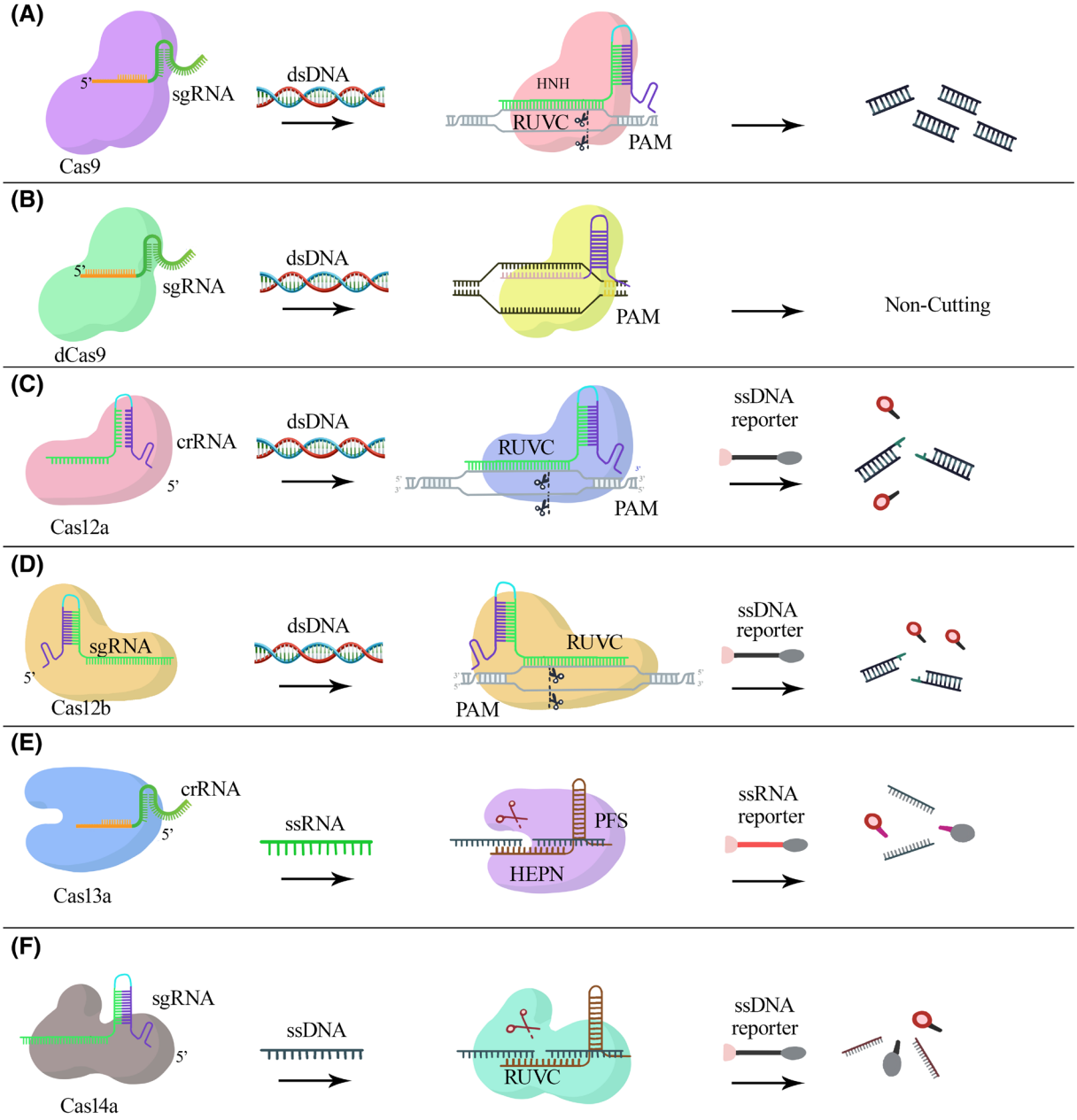

FIGURE 1 Diagrammatic representation of different Cas proteins and their cleavage abilities. (A) Cas9 requires two domains (HNH and RuvC) and produces blunt ends, (B) dCas9 catalytic activity mutated, (C) Cas12a needs PAM and targets dsDNA showing trans-cleavage activity, (D) Cas12b requires sgRNA like Cas9 but shows similar cleavage activity like Cas12a, (E) Cas13 mainly targets RNA, and (F) Cas14 do not need any specific PAM and target ssDNA.

to its simplicity, and high sensitivity but it may lead to false-positive results due to non-specific amplification. Loop-mediated isothermal amplification[59] (LAMP) operates at higher temperatures (65°C) and needs 4–6 complex primers, while strand displacement amplification[60] (SDA) is robust but insufficient for long-term transcripts. The exponential amplification method[61] (EXPAR) faces a large number of non-specific amplifications due to non-specific interaction between polymerase and synthetic DNA template. Likewise, the rolling circle amplification method[62] (RCA) provides rapid results but observes tricky detection, and nicking enzyme amplification[63] (NEA) cannot be used for intracellular and in vivo assays.

CRISPR–Cas-based platforms not only resolve the issue of false positive results caused by non-specific amplification through sgRNA recognition but also show exponential signal amplification ability[64] and the cost of CRISPR-based methods is lower than qPCR.[65] These amplification methods along with suitable biosensors can be used alone for pathogen detection but observed significantly lower sensitivity, specificity, and counter greater challenges than used with CRISPR technology. Therefore,

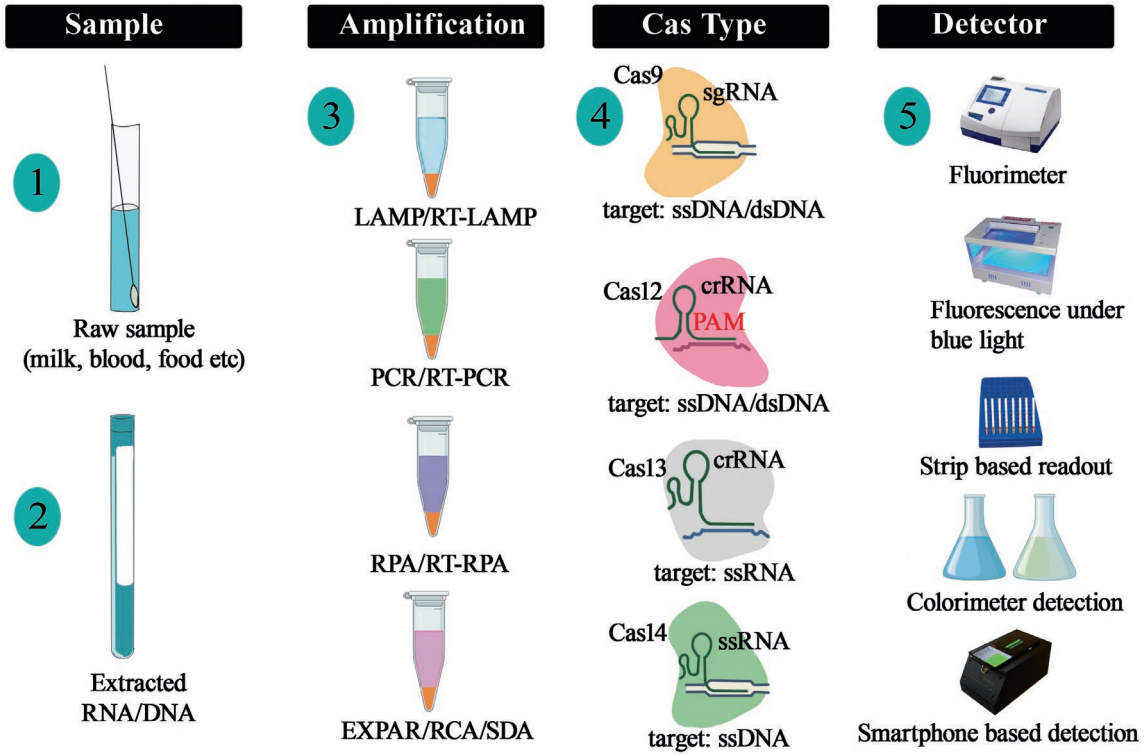

FIGURE 2 Graphical depiction of key steps in CRISPR–Cas systems for detecting infectious pathogens; (1) selection of sample, (2) extraction of DNA/RNA, (3) amplification of DNA/RNA product, (4) signal production by Cas proteins, and (5) biosensors to readout these signals.

combining these amplification methods with CRISPR-based biosensors have provided early pathogen detection platforms with high accuracy.[66]

4 | DIAGNOSTIC APPLICATION OF CRISPR SYSTEM IN ANIMALS

The rapid pathogen detection capability of the CRISPR/Cas system has stirred the scientific community to develop cost-effective point-of-care (POC) diagnostic methods for the control of human and animal diseases.[67] In this section, we have summarized (Figure 3) and highlighted the diagnostic ability of different CRISPR–CAS-based diagnostic platforms to rapidly identify the zoonotic and non-zoonotic bacterial, viral, parasitic, and other infectious diseases in pigs, ruminants, dogs and cats, poultry, and aquaculture.

4.1 | CRISPR–Cas system in pig diseases

African Swine Fever (ASF) caused by *ASF virus* (*ASFV*), is an acute and highly contagious disease of pigs, with a morbidity and mortality rate of reaching up to 100%.[68] *ASFV* causes huge economic losses to the swine industry and also poses a threat to food safety and global public health.[69] The genome of the dsDNA virus (170–190 kb in size) comprised of above 150 ORFs (open reading frames) and viral proteins (VP) and these (ORFs and VPs) play a key role in diagnosis and controlling the disease.[70] CRISPR–Cas system is widely used for *ASFV* detection. Wei et al. and Zhang et al. detected VP72[71] and D117L gene[72] of *ASFV* by utilizing the collateral cleavage activity of Cas13a and LwCas13a with a detection limit of only 10 copies and 2 copies, respectively. For clinical samples, both these tests showed a 100% coincidence rate with real-time PCR or qPCR. The gene amplification free assay, SERS combines Cas12a and magnetic beat to detect *ASFV* B646L gene with detection ranges from 100 nM to 10 fM.[73] Similarly, RAVI–CRISPR[74] and KP177R–RPA–CRISPR/Cas12a assay[75] utilizes rapid isothermal amplification techniques, LAMP and RPA, to detect *ASFV* genes (p72 and KP177R) within 35 and 30 min, respectively.

Porcine reproductive and respiratory syndrome (PRRS) causes severe economic losses to the pig industry and it is caused by a single-stranded enveloped RNA *PPRS virus*[76] of the family Arteriviridae. The droplet digital PCR (ddPCR) and qPCR tests used to diagnose *PRRSV* require long testing time along with the need for complex, expensive equipment, and trained operators. To overcome these issues, Liu et al. developed a fluorescent-based assay by

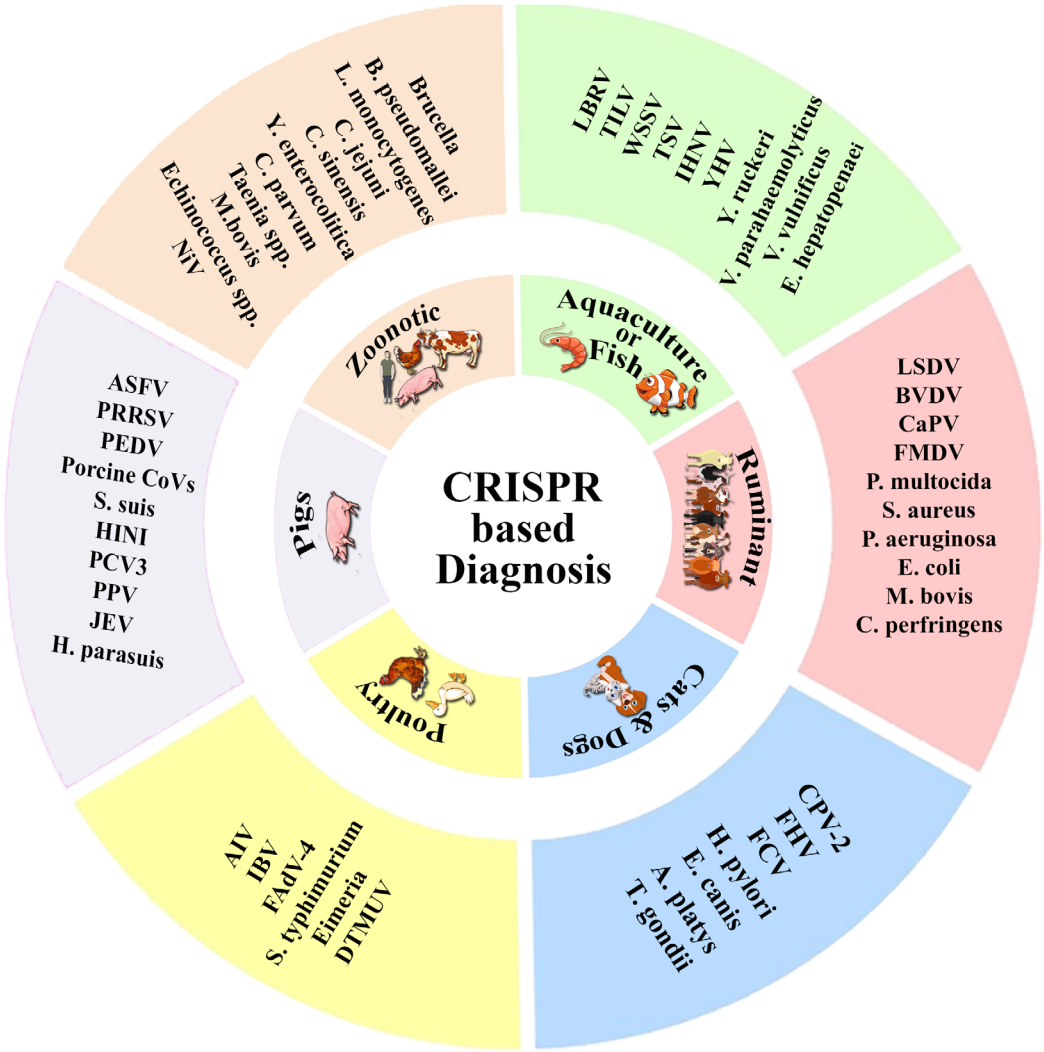

FIGURE 3 List of infectious pathogens diagnosed by CRISPR system in animals. CRISPR-based diagnostic platforms are used to diagnose infectious pathogens (viruses, bacteria, parasites, and protozoa) in different animals (aquaculture, ruminants, cats and dogs, poultry, and pigs), and disease of zoonotic potential.

targeting the nsp2 gene of *PRRSV* and combining reverse transcriptase RPA and CRISPR/Cas12 with a high sensitivity of 1 copy/reaction and a lower detection time of only 25 min.[77] The 11 clinical samples detected by CRISPR assay showed a highly accurate correlation with reverse transcriptase qPCR.

The severe enteric disease of unweaned piglets, first discovered in 1970 in the United Kingdom, Porcine epidemic diarrhea (PED), is caused by a member of the coronaviridae family.[78] The clinical symptoms of the disease include vomiting, diarrhea, and dehydration, but in severe cases lead to the death of the piglets in high numbers.[79] The structural protein, ORF3, and non-structural protein, spike, play an important role in virulence and viral attachment to host cells, respectively.[80] In 2021, Yang and Co targeted the early detection of structural protein (ORF3) to diagnose and differentiate wild and attenuated vaccines of *PEDV* using enzymatic recombinant amplification and CRISPR/CAS12 with LOD of only 2 copies per reaction.[81] One year later, the RAA–CRISPR–Cas12a–LFS method was developed to target the S gene of *PEDV* type II and differentiate *PEDV* type GI and GII with high sensitivity and specificity.[82] The 72 rectal swab samples were collected and examined using CRISPR assay and compared with qPCR. Both these tests reported a positivity rate of 55.6% with a 100% coincidence rate. The other enteric viral diseases caused by the member of coronaviridae include *transmissible gastroenteritis virus* (*TGEV*), *swine acute diarrhea syndrome coronavirus* (*SADS-Cov*), and *porcine delta-corona-virus* (*PD-Cov*).[83] These four viruses of the coronaviridae family were differentiated by a multiplex LAMP–Cas12a assay with high sensitivity of a single copy per reaction and signals were visualized by the naked eye using the colorimetric detection method.[84]

The small circular, single-stranded non-enveloped viruses of the Cicroviridae family cause a number of illnesses

in pigs.[85] Porcine dermatitis and nephropathy syndrome (PDNS), damage to lymphoid tissue, reproductive disorders, and immunosuppression are the highlights of this family.[86] Four types of closely related *Porcine circovirus* (*PCV*) species are *PCV1*, *PCV2*, *PCV3*, and *PCV4*. Lymph nodes clinical samples were collected by the Zhang group to specifically and timely diagnose *PCV-3* by a combination of ERA and CRISPR–Cas12 with the naked eye under UV light within 1 h.[87] Similarly, Wang et al. proposed the ultrasensitive CRISPR-based nucleic acid identification (RPA–CAS13a–LFD) system for the detection of *PCV-4* in serum samples and recognizing the *PCV-4* cap gene.[88] From 2018 to 2022, the outbreaks of diarrheal diseases in pigs in China led to the discovery of another type of virus called *Porcine Circovirus-like virus* (*PCLV*) that may be associated with *PCV*. Yu et al. detected the *PCLV* with high sensitivity (10 copies/reaction) on the basis CRISPR-based diagnostic system using isothermal amplification LAMP, with a match rate comparable to qPCR.[89]

The pig breeding industry is highly under the radar of *Porcine parvovirus* (*PP*V), the leading cause of infertility, stillbirth, and other reproductive disorders. Most of these illnesses were caused by *PPV-1* (discovered in Germany, 1965), while the incidence and prevalence of other species of the parvoviridae family, *PPV2-7* is very low and with no clear clinical signs.[90] Structural and the most important immunogenic protein, VP2, were targeted by ERA–CRISPR/Cas12a assay to successfully identify *PPV-1* with no cross-reactivity. Lateral flow dipsticks (LFD) can detect the targeted sequence and the whole process was completed in half an hour approximately.[91] The one-spot RAVI–CRISPR platform established by Xu et al. can be used to detect *Japanese Encephalitis virus* (*JEV*) with readouts either by the naked eye or fluorescence measurements.[92] *JEV* produces low levels of viremia and with no specific treatment available, this assay rapidly identifies the *JEV* C gene and can play a key role in preventing the spread of this zoonotic disease. Table 1 highlights the characteristics of different CRISPR platforms for pig diseases.

Glässer disease is caused by a non-motile, rod-forming, and gram-negative *Haemophilus parasuis* bacterium. This bacterium of the Pasteurellaceae family affects pigs of all age groups.[93] Taking benefits of the collateral cleavage activity of CRISPR–Cas12a, Zhang et al. and Hao et al. developed RPA–CRISPR/Cas12a assays. They used fluorescence biosensors to detect the *H. parasuis* Omp2 gene[94] and 16sRNA with a sensitivity of 0.163 pg/μL and 2.6 copies/μL,[95] respectively. The detection time of the later assay is almost half of the former with the results comparable to that of qPCR. Xu and co proposed a new method for the detection of Swine flu virus (*H1N1* virus) based on CRISPR–Cas13a. The CRISPR/Cas13a-assisted cascade amplification assay was based on ligation transcription and a series of amplification strategies. The fluorescence biosensor composed of three enzymatic reactions cleaves FAM molecules to detect fluorescent signals.[96] This method targeting RNA can be applied to detect the *H1N1* virus within 100 min with sensitivity reaching up to the picomolar level.

Rapid and ultrasensitive detection of contagious respiratory disease, Porcine pleuropneumonia, can be done using species-specific CRIPSR/Cas12a-assisted rapid detection platform.[97] This platform combines isothermal amplification and LbCas12a to detect *Actinobacillus pleuropneumoniae* apxIVA gene. This method designed by Luan et al. can be used in the field with a detection limit of 10 cfu without any cross reaction with other respiratory diseases. Chang et al. integrated LwCas13a (CRISPR) with T7 reverse transcriptase and RPA to develop a *porcine reproductive and respiratory syndrome virus* (*PRRSV*) detection method. The Enhanced Cas13a detection method can detect 172 copies/μL of the *PRRSV* M gene in 1 h. The enhanced Cas13a detection method can achieve a naked-eye readout by combining with LFD.[98]

Recently, Wang's group introduced a new CRISPR/Cas diagnostic tool known as MIRA–Cas12a based on a rapid MIRA and Cas12 protein. This technique identifies *pseudorabies virus* (*PRV*), the causative agent of Aujeszky's disease, and the results can be observed under blue light within 40 min.[103] In addition, MIRA–Cas12a also differentiates the vaccine and wild-type strains of *PRV*, targeting a number of genes TK, gE, and Gb with high specificity and sensitivity. Similar to this, Wei and group used a robust and portable *Streptococcus suis* detection method (Cards SSJ/K) based on the CRISPR/Cas12a system. This gram-positive bacterium can cross the blood–brain barrier and cause meningitis, arthritis, septicemia, and even death.[104] So, the Cards SSJ/K method can detect and differentiate *S. suis* serotypes (2 and 1/2) on the basis of cps2J and cps1/2K in less than an hour.

Senecavirus A (*SVA*) or *Seneca valley virus* (*SVV*) is a member of the Picronaviridae family and can cause fulminating contagious idiopathic vesicular disease in swine (SIVD). The symptoms of SIVD resemble those of foot and mouth disease and also with vesicular stomatitis.[107] Therefore, Ma et al. recently reported a POC detection of *SVV*. The RAA–CRISPR/Cas12a assay detected specific genes of *SVV* in clinical samples having signs of vesicular lesions with 100% accuracy. The specificity and sensitivity of the one-pot RAA–CRISPR/Cas12a method are comparable to that of RT-qPCR but the former detected 3D genes in a relatively shorter period of time.[105] Another important reproductive disease of sows and the causative agent of acute myocarditis in young pigs was detected by employing CRISPR–Cas13 in combination with RAA. The whole operation will be complete within an hour and

TABLE 1 Summary of different CRISPR platforms used in the diagnosis of pig diseases.

Disease	Pathogen	Amplification method	CRISPR/Cas system	Detection method	Limit of detection	Time	Target gene	Technique name	References
African swine fever	*ASFV*	PCR	Cas14	μPAD-based	5 copies/μL		dsDNA	CRISPR/Cas14a-G4	[99]
		RAA	Cas13a	LFS, VD	10 copies/μL	<1 h	p72	CRISPR/Cas13a-LFD	[71]
		RPA	LwCas13a	LFS	2 copies/ reaction	~50 min	D117L gene	RPA–LwCas13a–LFS	[72]
		RPA	Cas12a	FL or LFD	6.8 copies/μL	30 min	KP177R gene	KP177R RPA–CRISPR/Cas12a assay	[75]
		Amplification free	Cas12a	FL	~1 pM	90 min	B646L	Multiplex-crRNA CRISPR/Cas12a system	[100]
		Amplification free	Cas12a	SERS	100 nM to 10 fM	Within 2 h	B646L	CRISPR/Cas12a-SERS	[73]
		LAMP	Cas12a	NECR	7 copies/reaction	35 min	p72	RAVI–CRISPR	[74]
		PCR	Cas 12a	LFB	2.5×10^{-15} M	2 h	VP73	CRISPR/Cas–LFB	[101]
		PCR	Cas9 eraser	LFA			p72	CRISPR/Cas9 eraser	[102]
PRRS	*PRRSV*	RT-RPA	Cas12a	FL	1 copy	25 min	nsp2	CRISPR/Cas12a-based fluorescence detection	[77]
Porcine epidemic diarrhea	*PEDV*	RT-RAA	Cas12a	FL, VD under UV light, or LFS	100 copies	1.5 h	S	CRISPR/Cas12a-LFS	[82]
		RT-ERA	Cas12a	VD under LED blue light	2 copies	1 h	ORF3	RT-ERA-CRISPR/Cas12a detection	[81]
Porcine diarrhea	*Porcine CoVs*	Multiplex RT-LAMP	Cas12a	NECD	1 copy	25 min	ORF3, N	Multiplex LAMP–Cas12a assay	[84]
	PCLV4	LAMP	Cas12a	LFS	10 copies/reaction	60 min	ORF4	LAMP–CRISPR/Cas12a-based assay	[89]
PDNS	*PCV4*	RPA	Cas13a	LFD	1 copy/μL	1.5 h	PCV4 cap gene	RPA–CAS13a–LFD	[88]
	PCV3	ERA	Cas12a	Under UV/LED-blue light	7 copies	<1 h	Rep	ERA–CRISPR/Cas12a assay	[87]
Reproductive disorder	*PPV*	ERA	Cas12a	LFD	3.75×10^{2} copies/μL	~30 min	VP2	ERA–CRISPR/Cas12a system	[91]
Japanese encephalitis	*JEV*	RT-LAMP	Cas12a	NECR	8.97 copies	1 h	C	RAVI–CRISPR	[96]
Glässer	*H. parasuis*	RPA	Cas12a	FL	0.163 pg/μL	1 h	ompP2 gene	RPA–CRISPR/Cas12a	[94]
		RPA	Cas12a	FL	2.6 copies/μL	35 min	16S rRNA	RPA–CRISPR/Cas12a	[95]
Swine flu	*HINI*	T-7 cascade amplification	Cas13a	FL	3.23 pM	1 h 20 min	RNA	CRISPR/Cas13a-assisted cascade amplification	[96]
Porcine pleuropneumonia	*A. pleuropneumoniae*	RPA	Cas12a	FL	10 CFU	1 h	apxIVA	CARD	[97]
PRRS	*PRRSV*	RPA	Cas13a	LFS, FL	172 copies/μL	Within 1 h	M	Enhanced Cas13a detection	[98]
Aujeszky's disease	*PRV*	MIRA	Cas12a	Naked eye	28 copies/reaction	40 min	gB, gE, and TK	MIRA–Cas12a	[103]
Meningitis and arthritis	*S. suis*	RPA	Cas12a	FL	10 CFU	<1 h	cps 2J and cpc 2K	Cards-SSJ/K	[104]
SIVD	*Senecavirus A*	RPA	Cas12a	FL	10 copies	60 min	3D gene	RPA–CRISPR/Cas12a	[105]
Acute myocarditis	*EMCV*	RAA	Cas13a	LFS	10 copies/μL	Within 1 h	VP1	RAA–CRISPR/Cas13a assay	[106]

Abbreviations: ERA, enzymatic recombinant isothermal amplification; FL, fluorescence; LAMP, loop-mediated isothermal amplification; LFS/D, lateral flow strip/dipstick; MIRA, multi-enzyme isothermal amplification; NE, naked eye; NECR/D, naked eye colorimetric readout/detection; PCR, polymerase chain reaction; RPA, recombinant polymerization assay; VD, visual detection.

the output of the two-step based RAA–CRISPR/Cas13a assay[106] targeting the VP1 gene of *Encephalomyocarditis virus* (*ECMV*) can be visualized with the naked eye using lateral flow strips (LFS).

4.2 | CRISPR–CAS diagnostic application in ruminants

Ruminants (Cattle, Buffalo, sheep, and goat) play a key role in the progress of the livestock and food industry. They provide milk, meat, and a large number of other by-products made directly or indirectly from these animals. Any disease affecting these animals will pose direct harm to humans as well. So, the early diagnosis of these animal-related diseases will help to prevent and control the spread of diseases. In Table 2, specifications of CRISPR–Cas systems against livestock diseases have been discussed. The isothermal amplification method, RPA, is widely used to amplify nucleic acid but this technique has been under fire for a long time due to aerosol contamination.[108] Hao et al. provided a solution to avoid aerosol contamination during RPA amplification using round cover tubes. They also form a single pot CAS12a-NEye (naked eye) method by combining RPA with CRISPRCas12 protein to detect *Pasteurella multocida*. CAS12a-NEye assay[109] can detect the Kmt 1 gene of the causative agent of Bovine Hemorrhagic Septicemia and swine atrophic rhinitis in clinical samples within 1.5 h with high sensitivity.

In recent years, there has been a severe outbreak of Lumpy skin disease (LSD) in Cattle in Thailand, Pakistan, Indonesia, India, China, and many other Asian and European countries.[110] To minimize the spread and rapid diagnosis of *LSD virus* (*LSDV*), Liao et al. presented a CRISPR-powered platform.[111] With the sensitivity of only 1 copy/reaction, CRISPR-powered will identify *LSDV* in 90 min and also does not show any cross-reactivity with closely related *Capri pox virus* (*CaPV*) strains. Conversely, fluorescence-based RPA–CRISPR12a methods presented by the Jiang group against *LSDV* have further reduced the detection time to only 15 min.[112] Generally, PCR-based amplification methods require more than 1 h for amplification, but an ultra-fast PCR (UF-PCR) can complete the 40 cycles in approximately 15 min only. Combining UF-PCR with CRISRCas12 protein, Cao et al. made a field deployable *LSDV* detection system.[113] This naked eye visualization method detecting the B22R gene achieves the LOD of 20 copies/μL with highly comparable results to qPCR for 50 clinical samples. Additionally, the LOD of CRISPR/cpf1-mediated LFS diagnosis of *CaPV* was 1.47×10^3 TCID50 with 1000 times higher sensitivity over qPCR.[114] This LAMP-based method detected ORF001gene in mock and actual samples with a 100% coincidence rate.

More than 140 species were reported to cause mammary gland infection in animals, damaging animal productivity and posing significant economic losses to the dairy industry.[115] *Staphylococcus aureus* is one of the most common causes, accounting for 30%–40% of Mastitis in animals. Amanzholova et al. detected a gene of interest of *S. aureus* (nuc and sea) in mastitis-infected milk samples after coupling RPA and Lamp with *Moraxella bovis* Cas effector homolog, MbCas12a.[116] The resulting molecular RPA–CRISPR/Cas12a assay was completed in an hour with LOD of 10^4 and 10^2 copies per reaction, respectively. Similarly, Shaizadinova et al. also integrated MbCas12 with LAMP to detect the phoA gene in the plasmid, genomic DNA, and all 13 clinical isolates of *E. coli* from infected milk samples with high specificity.[117] Another strain of *E. coli* O157:H7 was detected from spiked meat samples in 30 min (after 4 h of enrichment) using fluorescence-based RAA–CRISPR/Cas12a detection method.[118] Likewise, *Pseudomonas aeruginosa* causing mastitis and other respiratory diseases in humans and animals were detected by CRISPR technology within an hour with a detection limit of 10 copies/μL.[119] The practical applicability of this RPA/Cas12a dual detection platform was observed in different food samples and showed highly consistent results with qPCR. Wand et al. proposed a novel one-pot OCTOPUS (RPA/Cas12a) platform with high precision and ultra-sensitivity at attomolar level for *Streptococcus aureus* and *E. coli* detection in milk samples.[120]

The spread of a disease can be prevented by an early-stage detection of nucleic acid. Yao et al. in vitro measured the RNA activity of LwCas13a and coupled a pair of crRNAs, quencher fluorescent RNA and LwCAS13a-protein to establish a CRISPR-LwCas13a-based detection system. This system can identify *Bovine Viral Diarrhea Virus* (*BVDV*) RNA with high sensitivity at pico-molar level in 60 min.[121] A Korean team of researchers also utilizes the collateral cleavage activity of the CRISPR-LwCas13a system to diagnose *BVDV* and target the *BVDV*-1b gene of single-stranded RNA virus.[122] The findings can be observed with the naked eye on lateral flow strips. To further reduce the detection time to only 15 min for *BVDV* with high sensitivity of 20 copies/μL, Wang et al. harmonized the enzymatic recombinant isothermal amplification method with CRISPR/Cas12a assay[123] and recognized similar results to qPCR for 112 clinical (anal or nasal) and 14 on-site field samples. Further to this, another enteric disease, hemorrhagic bowel syndrome can be rapidly identified using RAA–CRISPR/Cas12a-FL method constructed by Xiao et al. RAA–CRISPR/Cas12a-FL recognizes *Clostridium perfringens* cpa genes with a sensitivity cutoff of only two copies per reaction within a 1 h time frame.[124]

TABLE 2 Summary of different CRISPR setups used in diagnosis of ruminant diseases.

Disease	Pathogen	Amplification method	CRISPR/Cas system	Detection method	Limit of detection	Time	Target gene	Technique name	References
Bovine hemorrhagic septicemia	*P. multocida*	RPA	Cas 12a	NE	Single copy	Within 1.5 h	Kmt1 gene	Cas12a-Neye	[109]
Lumpy skin disease	*LSDV*	RPA	Cas12a	LFS	1 copy/reaction	90 min		CRISPR-powered platform	[111]
		RPA	Cas12a	FL	100 $TCID_{50}$/mL	15 min	orf068	RPA–Cas12a fluorescence assay	[112]
		PCR	Cas12a	FL or LFS	20 copies/μL	27 min	B22R gene	UF-PCR	[113]
Skin diseases	*CaPV*	LAMP	Cas12a	LFS	1.47×10^{-3} $TCID_{50}$	50 min	ORF001 gene	CRISPR/Cpf1-mediated LFS detection	[114]
Bovine mastitis	*S. aureus*	LAMP	MbCas 12a	FL	10^4 and 10^2 copies/reaction	1 h	nuc & sea	RPA–CRISPR/Cas12a	[116]
	E. coli	Lamp	Cas 12a	FL	13 copies	1 h	phoA		[117]
	P. aeruginosa	RPA	Cas 12a	LFTS	10 copies/μL	~30 min	lasB gene	RPA/CRISPR/Cas12a	[119]
	E. coli, S. aureus	RPA	Cas12a	FL	1 CFU/mL	<50 min	rfbE, nuc	OCTOPUS	[120]
	E. coli	RAA	Cas12a	FL	5.4×10^2 CFU/mL	30 min	wzy	RAA–CRISPR/Cas12a	[118]
Bovine viral diarrhea	*BVDV*	Amplification free	Cas13a	FL	1000 pM	1 h	RNA	LwCas13a-based detection system	[121]
		RT-PCR	LwCas13a	LFS			BVDV-1b gene	CRISPR–Cas13 system	[122]
		ERA	Cas 12a	FL	20 copies/μL	15 min	5′UTR gene	ERA–CRISPR/Cas12a	[123]
Hemorrhagic enteritis	*C. perfringens*	RAA	Cas12a	FL	2 copies/reaction	1 h	cpa gene	RAA–CRISPR/Cas12a-FL	[124]
Foot and mouth disease	*FMDV*	RT-ERA	Cas12a	FL	10 copies/μL	Within 30 min	RNA	RT-ERA–CRISPR/Cpf1	[126]
Anthrax	*B. anthracis*	SIBA	Cas 12a	FL	10^5–10^6 cfu	Within 40 min	Cya and Pag genes	CRISPR/SIBA	[127]
Yellow fever	*A. marginale*	RPA	Cas 12a	FL, LFD	4 copies/μL	Within 1 h	msp4 gene	RPA–CRISPR/Cas12a	[129]
East Cost Fever	*T. parva*	RPA	Cas 12a	LFS	1 infected lymphocyte/3 μL	80 min	p104	RPA–CRISPR/Cas12a	[130]
Neosporosis	*N. caninum*	RPA	Cas12a	LFS	1 parasite/mL	Within 90 min	Nc5 gene	RPA–CRISPR/Cas12a	[131]
Giardiasis	*G. duodenalis*	RPA	rCas12a	NE	10^{-1} copies/μL		β-Giardin gene	RPA–CRISPR/Cas12a	[132]

Abbreviations: ERA, enzymatic recombinant isothermal amplification; FL, fluorescence; LAMP, loop-mediated isothermal amplification; LFS/D, lateral flow strip/dipstick; NE, naked eye; NECR/D, naked eye colorimetric readout/detection; PCR, polymerase chain reaction; RPA, recombinant polymerization assay; SIBA, isothermal strand invasion-based amplification; VD, visual detection.

Foot and mouth disease (FMD) affects more than 70% of the livestock population globally. The listed and reported disease by the Office International des epizooties (OIE) in chronic cases leads to an almost 80% drop in milk production.[125] This severe and economic threat disease to the dairy and meat industry were rapidly diagnosed by Xiong et al. using CRISPR–CAS technology. They figure out the *FMD virus* (*FMDV*) with the threshold detection of 10 copies/μL, by amplifying the DNA using reverse transcriptase ERA (RT-ERA) and harnessing the trans cleavage activity of cpf1.[126] RT-ERA–CRISPR/Cpf1 assay diagnosed *FMDV* in 21 clinical samples within half an hour with 10 and 10^3 times more sensitivity over RT-qPCR and gel electrophoresis, respectively. The anthrax disease diagnostic platform CRISPR-SIBA, produced by adjoining a new isothermal strand invasion-based amplification (SIBA), Cas12 protein, and fluorescent-based readout method, can identify Prophage 3, cya, and pag gene of *Bacillus anthracis* within 40 min interval.[127] Patnaik et al. used a non-virulent strain *B. anthracis sterne* in CRISPR-SIBA diagnostic method to avoid any biosafety requirements.

Parasitic and protozoal causing diseases of ruminants like Yellow fever, East coast fever, Neosporosis, and giardiasis cause huge economic losses to the livestock industry. Recently, these diseases were rapidly diagnosed by an ultrasensitive CRISPR method. RNA-guided Cas12a enzyme and isothermal amplification RPA were combined in above mentioned disease diagnostic methods with different sensitivity outputs and readout strategies. Yellow fever caused by a parasitic bacterium *Anaplasma marginale* were transmitted to animals by tick vectors.[128] Patnaik et al. made a field deployable, visual readout, lateral flow dipstick recognition method targeting the msp4 gene of *A. marginale* with a detecting capability of 4 copies/μL.[129] Muruiki et al. developed a POC *Theileria parva* identification system and successfully detected the p104 gene in <1.5 h.[130] Similarly, the LOD of 1 parasite/mL for *Neospora caninum* was achieved by Wang et al. formed RPA–CRISPR/Cas12a assay to identify abortion causing protozoa in cattle.[131] Another protozoan infectious disease, Giardiasis (caused by *Giardia duodenale*), was rapidly identified by Zhao et al. When tested on clinically infected human and cattle fecal samples, the positive result output of this CRISPR-based platform was consistent with that of nested PCR, but had higher specificity and sensitivity.[132]

4.3 | CRISPR–CAS diagnostic application in dogs and cats

Pet dogs and cats are regarded as family members. Any disease affecting the pet's animals will harm other members of the family as well.[133] For almost half of a century, since its discovery in 1978, gastroenteritis caused by *canine parvovirus type 2* (*CPV-2*) is considered the main disease affecting mostly non-vaccinated puppies.[134] Haroon and co proposed an early onsite detecting CRISPR–Cas13a mediated nano-system for this high morbidity and mortality causing *CPV-2*. C2C2, also known as Cas13a, is an RNA-guided enzyme with a spacer region at 3′ end. When combined with targeted RNA and crRNA, this complex will produce non-specific degradation.[135] This collateral cleavage ability of Cas13a, along with rapid amplification RPA, and T7 transcription were used to develop CRISPR–Cas13a mediated fluorescent nano-system. Within a time span of 30 min (Table 3), this SHERLOCK-based method can detect *CPV-2* DNA at the atomolar level in a dog's intestinal samples.

Canine monocytic ehrlichiosis (CME) and Canine cyclic thrombocytopenia (CCT) were caused by *Ehrlichia canis* and *Anaplasma platys*.[136] Both of these intracellular bacteria were majorly transmitted by brown dog ticks and deer ticks and affect monocytes and platelets of the blood cells, causing anemia and thrombocytopenia, respectively.[136] These rickettsial pathogens were successfully detected by the RPA-assisted CRISPR Cas system developed by Paenkaew et al. In blood samples, this system showed 100 and 1000 times higher sensitivity over agarose gel electrophoresis for *E. canis* and *A. platys* accordingly.[137] Paenkaew et al. tailored systems have a detection limit of 100 copies and the whole process will be completed within 2 h. Another Cas12a and RPA coupled platform synthesized for skin and ear infection causing *Staphylococcus pseudintermedius* early diagnosis showed greater sensitivity than PCR. This Gao et al. designed visual readout setup will spot the spsJ gene in only 40-min duration.[138]

The minimum threshold for CRISPR–Cas12a-based optical *Helicobacter pylori* detection platform (COH assay) is only a single copy per reaction.[139] This gastrointestinal zoonotic pathogen identification system produced by integrating pre-amplification of targeted DNA by MIRA and trans-cleavage activity of Cas12a can be concluded in 45 min. COH assay can aid in the rapid detection of *H. pylori* ureA gene with more proficiency than a noninvasive urea breath test, stool antigen test, or an invasive rapid urease test. Additionally, Sun et al. manufactured a CRISPR and LFA-based medium and detected a positivity rate of 4%–8% for toxoplasmosis in cats and dogs, respectively, in Zhejiang province, China.[140] They selected *Toxoplasma gondii* B1 gene for identification and achieved an analytical detection limit of 31 copies/μL in stray dogs and cats. Further to this, Jiang and co. developed a single-tube setup called one-tube dRPA Cas12a/Cas13a assay to diagnose upper respiratory tract disease (URTD). This *feline herpes-1* (*FHV*) TK gene and *feline calicivirus* (*FCV*)

TABLE 3 Summary of different CRISPR systems used in diagnosis of dogs and cat diseases.

Disease	Pathogen	Amplification method	CRISPR/Cas system	Detection method	Limit of detection	Time	Target gene	Technique name	References
Hemorrhagic gastroenteritis	*CPV-2*	RPA	LwCas13a	FL	100 amol/L	Within 30 min	DNA	CRISPR–Cas13a-mediated nanosystem	[135]
Canine monocytic ehrlichiosis	*E. canis*	RPA	Cas12a	FL	100 copies	1.2 h	16 rRNA	RPA–CRISPR/Cas12a	[137]
Canine cyclic thrombocytopenia	*A. platys*	RPA	Cas12a	FL	100 copies	1.2 h	16 rRNA	RPA–CRISPR/Cas12a	[137]
	S. pseudintermedius	RPA	Cas12a	NE	10^4 copies/reaction	40 min	spsJ gene	RPA–CRISPR/Cas12a	[138]
	H. pylori	MIRA	Cas12a	LFS	1 copy/reaction	~45 min	ureA	COH assay	[139]
Toxoplasmosis	*T. gondii*	RPA	Cas12a	LFA	31 copies/μL	Within 55 min	B1 gene	RPA–CRISPR/Cas12a-LFA	[140]
URTD	*Feline herpesvirus, Feline calicivirus*	dRPA	Cas12, Cas13	FL	2.4×10^{-1} copies/μL, 5.5 copies/μL	50 min	TK gene, ORF1 gene	dRPA–Cas12a/Cas13a-Fluor assay	[141]

Abbreviations: FL, fluorescence; LFA/S, lateral flow assay/strip; MIRA, multi-enzyme isothermal amplification; NE, naked eye; NECR/D, naked eye colorimetric readout/detection; RPA, recombinant polymerization assay; VD, visual detection.

orf-1 gene dual pathogen recognition assay assembles the trans-cleavage activity of both Cas12a and Cas13a with dual recombinant amplification system (dRPA). The threshold sensitivity of one-tube dRPA Cas12a/Cas13a assay is 2.4×10^{-1} copies/μL and 5.5 copies/μL for *FHV* and *FCV*, respectively.[141]

4.4 | CRISPR–CAS diagnostic application in poultry

According to an estimate, there are more than 35 billion chicken birds in the world and a large number of these birds were affected by severe infectious disease outbreaks like Newcastle disease, Marek's disease, bird flu, infectious bronchitis, and many more.[142] They cause huge economic losses to the poultry and meat industry. A recent outbreak of *highly pathogenic avian influenza virus* (*HPAIV*), *H5N1* killed 82 million birds in America in 2022. To early detect the bird flu virus with on-site and visual eye capability, Zhou et al. coupled cpf1 protein with reverse transcriptase RPA.[143] The positive clinical results of the CRISPR–Cas12a assay showed slightly higher sensitivity than RT-qPCR and were indicated by a band at the test line on LFS or by a color change under blue light. Another important acute respiratory poultry disease associated with decreased egg production, infectious bronchitis, caused by *IB virus* (*IBV*), was rapidly observed by a lateral flow visual system based on SHERLOCK CRISPR–Cas13 (Table 4). The targeted spike gene of *IBV* was recognized within 50 min time frame without any cross-reactivity.[144]

The CRISPR-SERS was manufactured by Jia and group after the integration of highly sensitive analytical detective surface-enhanced Raman spectroscopy (SERS sensors) with CRISPR sensing system. The extracted DNA was directly triggered by CRISPR–Cas12a and after its trans-cleavage activity, the Raman signal reporter was activated and an intensity change of the Raman signal was observed.[145] This amplification-free platform will directly detect the targeted *Salmonella typhimurium* invA gene in 2 h. Moreover, Chang et al. assembled Cas12a protein with RPA isothermal amplification to identify seven *Eimeria* species (causing diarrhea, weight loss, and drop in egg production) by targeting SCAR (sequence characterized amplified region) marker genes. The fecal samples were first treated with aqueous solution of sodium hypochlorite and glass bead breaking to extract DNA, followed by RPA amplification and fluorescence detection system.[146]

The young broilers of age group 3–6 weeks were at higher risk of hepatitis hydropericardium syndrome (HHPS) caused by *fowl adenovirus* (*FAdV*). It can cause up to 30%–80% mortality in young flocks[147] and there is no

TABLE 4 Summary of different CRISPR systems used in the diagnosis of poultry diseases.

Disease	Pathogen	Amplification method	CRISPR/Cas system	Detection method	Limit of detection	Time	Target gene	Technique name	References
Avian influenza	*AIV*	RT-RPA	Cas12a	VE, LFS	1.9 copies/μL, 1.9×10^3 copies/μL	Within 1 h	H5 HA gene	CRISPR/Cas12a	[143]
Infectious bronchitis	*IBV*	RT-RPA	Cas 13	LFD	10 copies/μL	50 min	Spike gene	SHERLOCK	[144]
	S. typhimurium	Amplification free	Cas 12a	SERS	110 cfu/mL	Within 2 h	invA	CRISPR-SERS biosensor	[145]
Avian coccidiosis	*Eimeria*	RPA	Cas12a	FL	1 copy/reaction	>1.5 h	Scar markers	RPA–CRISPR/Cas12a	[146]
HHS	*FAdV-4*	RAA	Cas 13	LFA	10 copies/μL	Within 1.5 h	Hexon gene	CRISPR/Cas13- RAA	[148]
Tumbusu	*DTMUV*	RPA	Cas13a	LFD	1 copy/μL		E gene	CRISPR/Cas13a method	[149]

Abbreviations: FL, fluorescence; LFA/S/D, lateral flow assay/strip/dipstick; NE, naked eye; NECR/D, naked eye colorimetric readout/detection; RAA, recombinant assisted amplification; RPA, recombinant polymerization assay; SERS, surface-enhanced Raman scattering strategy; VD, visual detection.

effective treatment for serotype type 4 of *FAdv*. To prevent and early diagnose HHPS, Yin et al. used their previously proposed CRISPR/Cas13a-based recognition method.[148] This setup aided by isothermal amplification at 37°C and lateral flow visual output can detect the hexon gene of *FAdV* with a detection limit of 10 copies/μL. In 2022, Yin et al. developed the CRISPR/Cas13a system to identify the E gene of a new emerging *tumbusu virus* in breeding and laying ducks (*DTMUV*) causing a rapid drop in egg production.[149]

4.5 | CRISPR–CAS diagnostic application in aqua-culture

Fisheries and aquaculture play a vital role in our environmental stability and provide food and livelihood to a large number of the population. Infectious diseases affecting aquatic species, have caused huge economic losses in recent years. For example, white spot syndrome (WSS), Yellow head disease (YHD), and *Taura syndrome virus* (*TSV*) can lead to 100% mortality in penaeid shrimps spp. like pacific white shrimps, *Penaeus monodon*, and other aquatic species within a week. There are not any effective treatments for the double-stranded DNA, enveloped *WSS virus*. Aiming for early diagnosis to prevent *WSSV*, Chaijarasphong et al. synthesized CRISPR/Cas12 fluorescent assay.[150] Although this system requires less time to detect target genes but has a lower sensitivity (200 copies/reaction) than nested PCR (10 copies/reaction). Another, assay proposed by Sullivan et al. showed a greater threshold limit of only 1.06 (~1) copy per reaction.[151] This SHERLOCK-based CRISPR method coupled Cas13a enzyme with RPA to detect the *WSSV* VP108 gene (Table 5). In addition, RPA–CRISPR one-pot method for rapidly recognizing *WSSV*, developed by Wang et al. combined the amplification of targeted DNA and CRISPR–Cas12 protein trans-cleavage ability in one step reaction.[152] Furthermore, *TSV* SHERLOCK v2 assay, another single stage detection platform integrated LAMP with Cas12 to identify *TSV* VP1 gene in 30 min duration.[153] The binary qualitative results for 30 clinical samples, *TSV* SHERLOCK v2 assay showed 87% positive agreement with qPCR. RR–Cas system successfully catches the orf1b gene of genotype 1 of *yellow head virus* without any cross-reactivity with the other 8 genotypes of YHD but with lower sensitivity than nested RT-PCR in 30 clinical samples.[154]

Xinrui Lv et al. chemically modified the CRISPR/Cas12 and amplification method to avoid aerosol contamination and swiftly diagnose Early death syndrome or acute hepato-pancreatic necrosis disease (AHPND) in seafood.[155] CA-EE-CRISPR can spot *Vibrio parahaemolyticus* with an LOD of 73 CFU/g in shrimps. Derived

TABLE 5 Summary of different CRISPR platforms used in the diagnosis of aquatic diseases.

Disease	Pathogen	Amplification method	CRISPR/Cas system	Detection method	Limit of detection	Time	Target gene	Technique name	References
White spot syndrome	*WSSV*	PCR/RPA	LbCas12a	FL	200 copies	<1 h	VP28		[150]
		RPA	Cas13a	LFS	1.06 copies	Approx 1 h	VP108	SHERLOCK	[151]
		RPA	Cas12a	FL	10 copies/reaction	60 min	vp28	RPA–CRISPR one-pot method	[152]
Taura syndrome	*TSV*	LAMP	Cas12a	FL	10 copies/reaction	30 min	VP1	TSV SHERLOCKv2 assay	[153]
Yellow head disease	*YHV*	RT-RPA	LbCas12a	DNAzyme calorimetric readout	100 fg	1.5 h	orf1b	RR–Cas	[154]
Early death syndrome	*V. parahaemolyticus*	RAA	Cas12a	FL	73 CFU/g	<1 h	tlh gene	CE–RAA–CRISPR	[155]
AHPND	*V. parahemolyticus*	RPA	Cas12a	FL	100 copies/reaction	~90 min	pirB gene	RPA–CRISPR one-pot assay	[156]
	V. vulnificus	RPA	Cas12a	FL	4 copies/reaction	40–60 min		One-step RPA–CRISPR assay	[157]
Hepatopancreatic microsporidiosis	*E. hepatopenaei*	RPA	Cas12a	FL	10 copies/reaction	40 min	swp gene	One-pot RPA–CRISPR detection assay	[158]
Body surface ulcer syndrome	*LMBV*	RT-RAA	Cas13a	FL	31 copies/μL	1 h	MCP gene	CRISPR/Cas13a	[159]
Enteric redmouth disease	*Y. ruckeri*	RPA	Cas13a	LFA	2 aM	<70 min	gyrA gene	CRISPR/Cas13a system	[160]
Infectious hematopoietic necrosis	*IHNV*	RT-RPA	Cas12a	NE under UV light	9.5 copies/μL	Within 1 h	N gene	RT-RPA-CRISPR/Cas12a	[161]
Syncytial hepatitis	*TiLV*	RT-RAA	Cas12a	FL	9 copies	~1.5 h	S3	CRISPR/Cas12a system	[162]

Abbreviations: FL, fluorescence; LAMP, loop-mediated isothermal amplification; LFS, lateral flow strip; NE, naked eye; NECR/D, naked eye colorimetric readout/detection; RAA, recombinant-assisted amplification; RPA, recombinant polymerization assay; VD, visual detection.

from a suboptimal PAM of Cas12a, Wang et al. designed a specially structured crRNA and assembled it with RPA amplification and CRISPR system to identify *V. parahaemolyticus* pirB gene simultaneously in one reaction.[156] Likewise, other seafood affecting vibrio species (*V. vulnificus*) were analytically observed by one-step RPA–CRISPR assay in 40–60 min with a sensitivity threshold of 4 copies per reaction.[157] In the same way, one-phase fluorescent-based CRISPR detection platform can diagnose hepatopancreatic microsporidiosis within 40 min to prevent the drop in production in shrimp population by 10–20% every year due to *Enterocytozoon hepatopenaei*.[158] This one pot-RPA assay has an LOD of only 10 gene copies and detected 16/20 positive shrimp samples with 100% consistency to nested PCR.

Since its introduction into China in the 1980s, one of the favorite Chinese people's fish, Largemouth bass (*Micropterus Salmoides*), were now cultured in many parts of China. This fish is severely affected by *Largemouth bass ranavirus* (*LMBV*), discovered in 1995, with a mortality rate of 70%. Guang et al. selected the conserved region of the *LMBV* MCP gene for targeting by crRNA and developed a CRISPR/Cas13 rapid detection system with fluorescence-based visual readout under UV light.[159] Similarly, the CRISPR/Cas13 system developed by a team from Chile can early diagnose enteric redmouth disease in omega 3 rich fatty acids containing Salmon fish. This method demonstrated the pinpoint identification of *Yersinia ruckeri* gyrA gene from planktonic and biofilm samples with sensitivity comparable to RT-qPCR.[160] Recently, the CRISPR/Cas12a assay was employed to detect *infectious Hematopoietic necrosis* (*IHN*) *virus*, another wild salmon damaging disease, and achieved an LOD of 2 aM.[161] In addition, *the tilapia lake virus* detection platform, combines reverse transcriptase RAA, Cas12a, and modified ssDNA reporters, and detects as few as 9 copies per reaction to early diagnose and prevent further economic losses to the aquaculture industry.[162]

4.6 | CRISPR–CAS diagnostic application in zoonotic diseases

Zoonotic diseases are transmitted from animals to humans or humans to animals. According to a report by WHO, almost 60% of diseases in humans are zoonotic and an estimated 75% of the newly emerging diseases are zoonotic. These diseases are transmitted by aerosol transmission, either through direct contact with infected animals or eating contaminated meat, food, or drinking unpasteurized milk. Veterinarians, farm workers, butchers, and laboratory workers are at higher risk of being infected by zoonotic pathogens than other human beings.[163] *Yersinia enterocolitica* is an important zoonotic gram-negative bacteria and can cause Yersiniosis in humans, pigs, and ruminants. An RPA–CRISPR/Cas12a-based identification method has been demonstrated by Xiao et al., for the detection of the *ail* gene of *Y. enterocolitica* in artificially contaminated pork meat with 100 times greater sensitivity over qPCR.[164] Similarly, spiral-shaped gram-negative bacteria and one of the most common food-borne pathogens in the United States, *Campylobacter jejuni* were rapidly identified by RAA–CRISPR/Cas12a method. This system based on a field fluorescent reader visualization system detected 5 copies of the hipO gene of the *C. jejuni* strain in spiked chicken and meat samples.[165]

Globally, an important food-borne zoonotic pathogen, *Listeria monocytogenes* causes Listeriosis, both in human and animal populations with high mortality rates. The micro-propulsion reactor system, Cas12a-MPR, not only detected *L. monocytogenes* with high specificity in milk samples but also highlighted the importance of magnesium ions in CRISPR/Cas12a system.[166] Furthermore, two *L. monocytogenes* detection methods, micro-amplification platform (Cas12-MA)[167] and RAA–CRISPR/Cas12a,[168] integrated Cas12a protein with RPA and RAA isothermal amplification and recognize hly gene in meat samples within 25 min and 35 time frame, respectively (Table 6). Although many countries of the world were declared Brucella free, it is still highly prevalent in cattle and sheep populations in developing countries. There is no effective vaccine for humans and imposing a culling policy on infected animals, it poses an economic burden to farmers. Early on-site detection with CRISPR/CAST[169] or F/E-CRISPR system[170] in serum/milk samples with high specificity can aid in preventing the spread of the disease. The blood samples of 398 sheep and 100 cattle were tested by CRISPR/CAST package and showed 7.7% and 8% positivity rates with detection rates comparable to qPCR. Recently, Wang et al. (2024) presented a highly efficient molecular detection platform (RPA–CRISPR–Cas13a–LFD) to detect Bovine tuberculosis, another respiratory disease of significant importance that can be transmitted to human by consuming unpasteurized milk and eating contaminated meat. In milk samples, the MPB 870 gene of *Mycobacterium bovis* was detected by RPA–CRISPR–Cas13a–LFD assay with LOD of only a single copy per reaction.[171] This method can be applied to early diagnose *M. bovis* and lower the spread of the disease in developing countries.

Zhang et al. developed a 40 min, low-cost CRISPR–Cas12a system, which includes isothermal RPA amplification and identification of specific tags by Cas protein[172] to diagnose sepsis and organ dysfunction causing Melioidosis. This in-vitro method can detect bacillus *Burkholderia pseudomallei* LC1 and LC2 specific tags,

TABLE 6 Summary of different CRISPR systems used in diagnosis of zoonotic diseases.

Disease	Pathogen	Amplification method	CRISPR/Cas system	Detection method	Limit of detection	Time	Target gene	Technique name	References
Yersiniosis	*Y. enterocolitica*	RPA	Cas12a	FL	1.7 CFU/mL	45 min	ail	RPA–CRISPR/Cas12a	[164]
Campylobacteriosis	*C. jejuni*	RAA	Cas12a	FL	5 copies	30 min	hipO	RAA–CRIPSR/Cas12a	[165]
Listeriosis	*L. monocytogenes*	RPA	Cas12a	FL	10 CFU/mL	45 min	hly	Cas12a-MPR	[166]
		RPA	Cas12a	FL	4.4 CFU/g	25 min	hly	Cas12a-MA	[167]
		RAA	Cas12a	FL	350 cfu/mL	35 min	hly	RAA–CRISPR/Cas12a	[168]
Brucellosis	*Brucella*	RPA	Cas12a	FL or LFS	10 copies/μL	30 min	bp26	CRISPR/CAST	[169]
		RPA	Cas12a	FL and EC biosensor	2 copies/reaction	30 min	omp2a	F-CRISPR, E-CRISPR	[170]
Bovine tuberculosis	*M. bovis*	RPA	Cas13	LFD	2 copies/μL	2 h	MPB 870	RPA–CRISPR–Cas13a–LFD	[171]
Melioidosis	*B. pseudomallei*	RPA	Cas12a	FL	0.2 & 2 copies/reaction, respectively	<40 min	LC1 & LC2	CRISPR–Cas12a	[172]
	C. parvum	RPA	Cas12a	LFS biosensor	Single copy	~1 h	GP60	ReCTC	[173]
CCA	*C. sinensis*	RPA	Cas12a	LFS	1 copy/μL	Within 1 h	pasteu	RPA–CRISPR/Cas12a	[174]
Toxoplasmosis	*T. gondii*	RPA	LbCas12a	FL or LFS	3.3 copies/μL	~50 min	B1	RPA–CRISPR/Cas12a assay	[175]
		RAA	Cas12a	FL	1 fM	~1 h	RE	RAA–Cas12a-Tg system	[177]
		RAA	Cas13a	LFD	1×10^{-6} ng/μL	Within 2 h	B1	RAA–Cas13a–LFD assay	[178]
Avian influenza	*AIV*	RAA	Cas13a	LFD	1 copy/μL	~1 h	NP gene	RAA–CRISPR–Cas13–LFD	[176]
Metacestodiasis	*Taenia spp and Echinococcus spp*	RCA	Cas 9	FL	10 aM	2.5 h	miRNA let-7-5p	RCA-assisted CRISPR/Cas9	[179]

Abbreviations: FL, fluorescence; LFS/D, lateral flow strip/disk; NE, naked eye; NECR/D, naked eye colorimetric readout/detection; RAA, recombinant-assisted amplification; RCA, rolling circular amplification; RPA, recombinant polymerization assay; VD, visual detection.

with high sensitivity threshold. Subsequently, Yu et al. employed the collateral cleavage activity of the Cas12 enzyme and RPA amplification to form the ReCTC system to control, Cryptosporidiosis, the second greatest cause of diarrhea. This identification tool rapidly detects *Cryptosporidium parvum* in human and cattle fecal samples with a detection limit of only a single copy.[173] *Clonorchis sinensis*,[174] a zoonotic food-borne parasite that resides in the liver and bile duct and risk factor for cholangiocarcinoma was diagnosed by the RPA–CRISPR/Cas12a system. This dual read-out platform detected pasteu genes of group I classified biological carcinogen in human swab and fish flesh samples with a positivity rate comparable to nested PCR; 10% (5/50) and 28% (14/50), respectively. Moreover, Cas12/Cas13 proteins with RPA and RAA amplification systems have also been used to diagnose *T. gondii*[175] and *AIV*.[176] These units targeted B1/RE and NP genes of respective pathogens and the whole process was completed within an hour.

5 | CRISPR–CAS APPLICATIONS

Besides clinical and infectious diseases diagnostic application of the CRISPR–Cas system, CRISPR technology holds great importance in its application in genetic engineering,[180] gene editing/mutation in human cells to treat various human disorders like HIV,[181] and hematological disorders (thalassemia[182] and sickle cell anemia[183]). The CRISPR tool is paramount in gene regulation, producing pharmaceutical proteins, and investigating gene function.[184] It exhibits remarkable usefulness in plant breeding[185] for the enhancement of different attributes including quality, yield,[186] nutritional factors,[187] and crop improvement[188] like disease resistance,[189] drought tolerance,[190] desirable traits,[191] and development of crop in low time. CRISPR technology offers considerable potential for bio-sensing,[192] food safety,[193] food and agricultural sector, environmental monitoring,[194] biosafety analysis,[195] and biofuel production.[196] The CRISPR genetic modification ability has been used in animal breeding for developing animals with enhanced disease resistance,[197] and in animal models[198] for enhancing animal health, welfare, and production.[184] In the field of oncology, CRISPR/Cas9 is mostly used to target and modify cancer-related genes,[199] treatment, and therapeutics using miRNA therapy.[200,201] CRISPR's importance for drug discovery and gene therapy[202] (Casgevy: an FDA-approved therapy for sickle cell disease) and for early detecting, diagnosing, and treatment of non-infectious diseases like heavy metals[203] is undeniable. Figure 4 highlights the application of CRISPR technology is various fields.

6 | CONCLUSION AND FUTURE PERSPECTIVE

Animal and zoonotic diseases like Avian influenza, COVID-19, tuberculosis, and brucellosis, pose a severe threat to the survival of animals and public health. It is therefore need of hour to early detect the pathogens to reduce mortality, morbidity, and to control and prevent the disease. Among the currently available diagnostic methods, CRISPR–Cas systems provide a rapid, ultrasensitive detection platform. Pathogen culture and qPCR, the gold standard diagnostic test of many diseases, are slow and also require the need of costly laboratory equipment. In this review, we overviewed recent breakthroughs in diagnosing infectious diseases in animals (pigs, ruminants, cats and dogs, poultry, and aquaculture) and diseases of zoonotic importance based on the CRISPR–Cas platform. The reported diagnostic system, CARD, Cas12-MA, COH assay, RR–Cas, and CRISPR/CAST have been reported to detect *A. pleuropneumoniae*, *LSDV*, *H. pylori*, *YHV*, Brucella in pigs, cattle, dogs, aquaculture, and human/cattle, respectively with greater sensitivity. The classification and unique characteristics of Cas proteins have also been demonstrated. Cas 9 only depicts cis activity while Cas12, Cas13, and Cas14 enzymes exhibit collateral cleavage activity with and without the need for specific PAM, respectively. Despite the fact, isothermal-based amplification (RPA and LAMP) techniques have certain drawbacks like the need for more complex primers for LAMP, aerosol contamination, and no appropriate primer design software for RPA. They have more advantages over PCR and are widely used in animal diagnosis. Adjoining CRISPR–Cas and amplification strategies have drastically lowered the detection time from days to hours and hours to minutes, with other benefits like onsite detection and high specificity.

However, the CRISPR-based diagnostic platforms face a lot of challenges for on-field POC testing including the inability to direct testing of raw samples, pre-treatment of samples for nucleic acid extraction,[204] and the off-target effects[205] caused by Cas effector and mismatch between sgRNA and the target. The lack of standard reaction (temperature, pH, and ion concentration) and storage conditions for an ideal platform,[66] complexities associated with multiplexing[21]; unspecific trans-cleavage activities, dependence on labels for signal generation and the risk of cross-contamination.[206] The portability and automation of biosensors still need a lot of in-depth research to overcome these obstacles. Other than these, mostly these animal-related CRISPR platforms target majorly nucleic acid for detection, but limited work has been done on

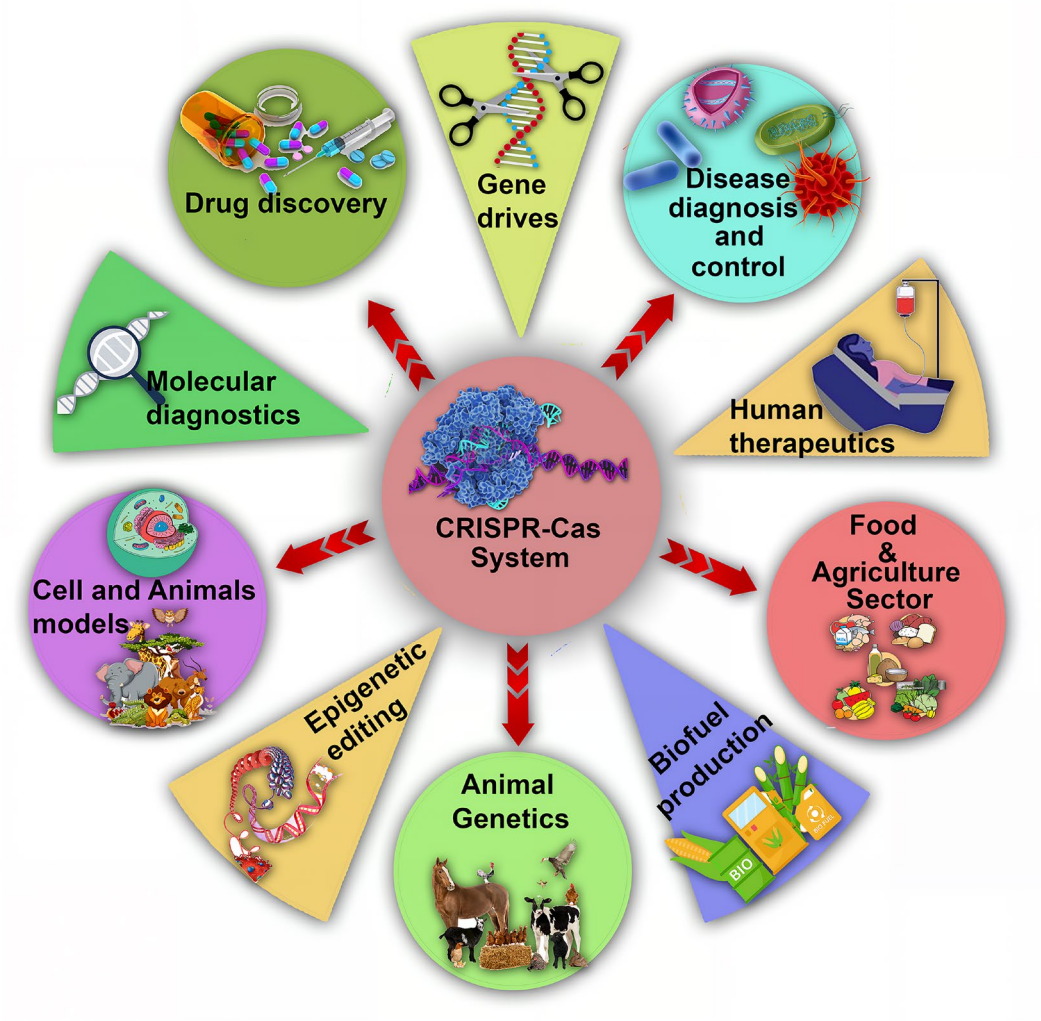

FIGURE 4 CRISPR–Cas systems: versatile applications. CRISPR–Cas systems are transforming multiple fields, from precise gene editing in healthcare to rapid diagnostics and agricultural advancements. Their ability to target and modify DNA makes them powerful tools for both research and real-world solutions.

targeting other analytes[207] (protein, small molecules, exosomes, etc.) using aptamers,[208] allosteric transcriptional factors,[209] riboswitches, and/or DNAzyme.[210]

Moreover, the detection of animal or zoonotic pathogens counter greater challenges than human clinical samples like the pretreatment method for samples is quite difficult due to the complex and inconsistent nature[211] (milk, meat, and feces) of samples. Also, few relevant studies reported lower sensitivity than qPCR and nested PCR.[150] Therefore, it can be assumed that in the future more CRISPR setups will be developed with atomolar threshold levels like SHERLOCK, CARVER,[212] and with different sample-type screening capabilities. Secondly, aerosol contamination during RPA-based methods can be avoided by employing single-pot strategies like Cas12-MA[167] and Cas12a-NEye.[109] Thirdly, mostly developed diagnostic platforms focused on collateral cleavage activity of Cas12/Cas13, but one might predict more systems like CCIP[213] and SPCas[33] will be designed using PAM-free collateral cleavage potential of Cas14. Fourth, more amplification-free systems[66] will be formed to further lower the cost of reagents and other instruments. Lastly, more emphasis will be given to developing multiplex pathogen detection units to detect various pathogen targets in a single reaction tube.[214] Despite the fact, a lot of CRISPR detection methods have been reported against animal diseases but these platforms need to be put into tool boxes for real-world use (clinical and resource-limited areas) with free nucleic acid extraction detection. Therefore, merging these aspects and future cutting-edge research will further lead to the development of ideal CRISPR–Cas detection platforms. Ahead of curve approach to hurdles, nurturing new ideas, and adapting new technologies will shape the future of the CRISPR system, transform disease diagnosis capability and improve human and animal health.

AUTHOR CONTRIBUTIONS

All authors contributed to the study's conception and design. Hafiz Muhammad Hamza Rasool made the primary draft, designed the figures, and completed the manuscript. Qiwei Chen, Xiaowei Gong, and Jizhang Zhou provided guidance and help to revise the manuscript. All authors read and approved the manuscript.

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DISCLOSURES

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

All relevant data are contained within the article and data sharing is not applicable to this article as no new data were created or analyzed in this study.

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