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REVIEW ARTICLE

Recombinase Polymerase Amplification for Rapid Detection of Zoonotic Pathogens: An Overview

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Abstract

With the advent of molecular technology, several isothermal techniques for rapid detection of zoonotic pathogens have been developed. Among them, recombinase polymerase amplification (RPA) is becoming an important technology for rapid, sensitive, and economical detection of zoonotic pathogens. RPA technology has the advantage of being able to be implemented in field settings, because the method requires minimal sample preparation and is performed at a constant low temperature (37–42°C). RPA is rapidly becoming a promising tool for the rapid detection, prevention, and control of zoonotic diseases. This article discusses the principles of RPA technology and its derivatives, including RPA coupled with lateral flow testing (RPA-LF), real-time fluorescence RPA, electrochemical RPA, and flocculation RPA, and their applications in the detection of zoonotic pathogens.

Key words: recombinase polymerase amplification, rapid detection, zoonotic pathogen, zoonosis, RPA

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INTRODUCTION

Nucleic acid amplification (NAA) in vitro, the artificial replication of genetic material, has permeated nearly every field of the life sciences and biotechnology. This technology originated from the invention of polymerase chain reaction (PCR) by Kary Mullis in 1983 [1]. The PCR amplification technique has been widely used in the rapid detection of nucleic acids and demonstrated to be highly specific and efficient. However, the PCR amplification technique has several limitations. First, it is somewhat limited by its reliance on a thermal cycler for heating and cooling, and high-quality nucleic acid reactants. Furthermore, skilled operators and a laboratory environment are also needed, thus preventing its application in low-resource settings.

To overcome the limitations of PCR methods, multiple isothermal DNA amplification methods using various enzymes and amplification systems have been established, including loop-mediated isothermal amplification (LAMP), rolling circle amplification (RCA), nucleic acid sequence-based amplification (NASBA), helicase-dependent amplification (HDA), strand displacement amplification (SDA) and recombinase polymerase amplification (RPA) (Table 1) [2–4]. Isothermal NAA greatly simplifies the incubation conditions for artificial NAA, and the elimination of thermal cycling decreases the need for amplification equipment or a laboratory environment. Another advantage of the isothermal NAA is that multiple molecular reactions, such as denaturation, annealing, and elongation, can be performed asynchronously in one isothermal amplification reaction, thus effectively decreasing the NAA reaction time [5,6]. Among all

TABLE 1 | Isothermal nucleic acid amplification techniques.

Isothermal technique	Template	Primers	Temperature (°C)	Incubation time (min)	Lyophilized reagents
LAMP	DNA	4~6	60~65	15~60	No
RCA	DNA/RNA	1	30~65	60~240	No
NASBA	RNA	2	37~42	60~180	Yes
HDA	DNA	2	60~65	30~120	No
SDA	DNA	4	30~55	60~120	No
RPA	DNA/RNA	2	37~42	20~40	Yes

isothermal DNA amplification methods, RPA is notable for its simplicity, and high specificity and sensitivity, despite its very short history of use.

RPA

RPA was first developed by Niall Armes at ASM Scientific Ltd of Cambridge, UK in 2006 [7]. Unlike traditional PCR methods, RPA does not rely on thermal denaturation and annealing. Three key proteins required for RPA reactions are a recombinase that binds single-stranded nucleic acid, a single-stranded DNA binding protein, and strand-displacement DNA polymerase [7,8]. The amplification reaction starts with the binding of the recombinase to a primer of approximately 30–35 nt, and the complex's subsequent search for the target site in the double-stranded DNA template. After the site is located, the complex directly initiates a chain exchange reaction to form a D-shaped loop. Single-stranded binding protein then binds the displaced DNA chain to prevent primer dissociation. Subsequently, the active hydrolysis of ATP by the recombinase-primer complex alters the conformation of the complex. After the recombinase releases from the nucleoprotein filament, the 3′ end of the primer is exposed and recognized by the strand-displacement DNA polymerase. The DNA polymerase adds the corresponding base to the 3′ end of the primer according to the template sequence, and the DNA amplification reaction begins. Both forward and reverse primers allow the amplification reaction to occur in both directions simultaneously. The synthesized amplicon can be used as a new template to achieve exponential amplification Figure 1 [9].

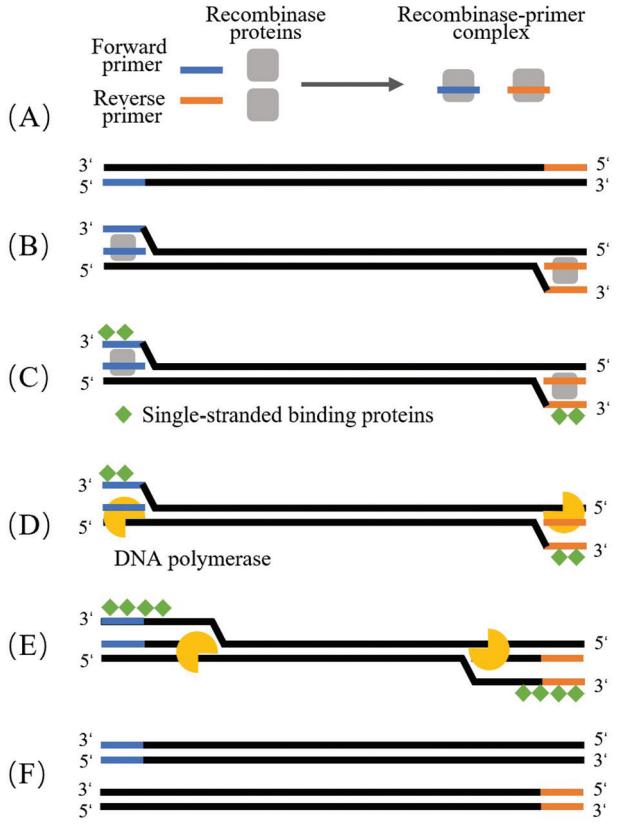

FIGURE 1 | Schematic of the RPA principle. (A) The recombinase proteins bind each primer. (B) The complex searches for the target site and directly initiates a chain exchange reaction, forming a D-loop. (C) Single stranded binding proteins bind the displaced DNA chain, thus preventing primer dissociation. (D) The recombinase releases the nucleoprotein filament, and the DNA polymerase extends the primer 3′ ends. (E) The DNA amplification reaction starts. (F) Exponential amplification is achieved by cyclically repeating the process.

Template

RPA was initially designed to amplify double-stranded DNA, single-stranded DNA, and DNA methylation [10,11]. The cDNA produced by reverse transcription of RNA or miRNA can also be amplified [12,13] in a process requiring reverse transcriptase [14]. Regardless of the nucleic acid template type, the length of the RPA amplicon should be less than 500 bp for efficient amplification. Most published articles on RPA have shown that although targets up to 1.5 kb can be amplified, this method is more suitable for amplicons of between 100 and 200 bp [7,15], because shorter the sequences result in higher amplification efficiency.

Primers and probes

In contrast to traditional PCR primers, RPA primers are generally 30–35 nucleotides long, to facilitate the formation of a complex between the recombinase and the primer. Longer primers (up to 45 nucleotides) may produce secondary structure and potential primer artifacts, thus decreasing amplification efficiency. In addition, no melting

temperature requirement exists for RPA primer and probe design. Like traditional PCR primers, RPA primers should have a GC content of 30%–70%, and long-chain guanines should be avoided at the 5′ end, whereas guanine and cytosine nucleosides can be used at the 3′ end to improve performance. A probe is not necessary in standard RPA assays but is required when RPA is combined with the various endpoint detection methods described below [16–18]. The procedure for primer and probe design is not standardized, and no dedicated software is available; however, the selected primers and probes can be evaluated with software for PCR primer design, such as Primer Premier 5. Multiple groups of primers and probes are usually designed and evaluated in experiments to screen for the best combinations [19–22].

Temperature and incubation time

The optimum reaction temperature of the enzymes in RPA assays is 25–42°C; thus, the optimum temperature for the RPA reaction is also in this range. RPA assays do not require strict temperature control [23–25]. Even if the temperature exceeds the recommended range, the RPA reaction can still proceed. However, the recommended RPA reaction temperature in most studies is 37–42°C [9,26].

The time required for NAA to reach a detectable level depends on the concentration of the starting DNA template. At the appropriate reaction temperature, detection can be completed within 20 minutes [27]. In practical applications, the amplification results can be observed in as few as 3–4 minutes. For solution phase RPA amplification reactions, the recombinase can consume all ATP in the system within 25 minutes; therefore, long incubation times are unnecessary. In addition, a shaking step at the fourth minute of the reaction is recommended to increase reaction efficiency [20,21,28].

Advantages and disadvantages of RPA assays

RPA assays offer several advantages. NAA can be performed under constant temperature conditions of 37–42°C without pre-denaturation steps and high temperature annealing steps. Therefore, RPA does not require expensive thermal cycling equipment and is suitable for non-instrumented NAA platforms. Researchers have also used human body temperature to complete the amplification in various conditions [28].

RPA technology is simple to perform and has good expansibility, and can be combined with various systems for different detection purposes. Combining this method with reverse transcriptase in reverse transcription-RPA (RT-RPA) can be used to amplify RNA sequences; combining RPA with a fluorescence probe in real-time fluorescence RPA can achieve real-time detection; and combining RPA with lateral flow (LF) in RPA-LF can detect enable visual target sequence detection with the naked eye [18,29–32]. Recently, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nuclease (Cas) have been used to develop a CRISPR-based diagnostic method called SHERLOCK, thus increasing the popularity and scope of application of RPA assays [33].

RPA reagents can also be used in lyophilized forms with excellent stability, and be transported and stored without refrigeration [34,35]. Multiplex RPA assays that can detect multiple target sequences quickly in a single reaction are also available, depending on the target sequence, amplicon size, and primer design [36–38]. All these advantages facilitate the implementation of RPA in field-based rapid detection applications. In recent years, RPA methods have been extensively developed for the rapid detection of various pathogens, particularly zoonotic pathogens.

Some shortcomings of RPA technology include that RPA products normally require purification before agarose gel electrophoresis to avoid smearing due to the presence of other components. Second, no specialized software is available for the design and screening of RPA primers and probes. Thus, costly and time-consuming synthesis and screening studies are required. Furthermore, conventional real-time PCR probes (such as Taq-Man probes) are not compatible with the RPA reaction, and fluorescent dyes are prone to false positive results. In addition, real-time amplification with RPA is not easily controlled, owing to its isothermal amplification properties and the use of a time threshold rather than a cycling threshold. Thus, the reactions are dependent on the initial conditions, incubation temperature, and mixing steps. With regard to cost, RPA kits are currently sold by only one company, and users have limited flexibility in the kit formulations; consequently, small batch use has high costs.

APPLICATIONS OF RPA IN ZOONOTIC PATHOGEN DETECTION

Zoonoses are any diseases or infections that are naturally transmissible from vertebrate animals to humans, or from humans to animals [35,39]. Approximately 60% of emerging human infections are zoonoses, and more than 70% of pathogens originate from wild animal species [40]. At present, more than 200 species of zoonotic pathogens are known worldwide, the most prevalent of which are anthrax, plague, foot-and-mouth disease, avian influenza, Japanese encephalitis, and rabies [41–43]. Zoonoses are a major public health concern and directly threaten human health. In recent decades, large-scale epidemics of zoonoses have occurred, such as the 2005 H5/N1 avian influenza outbreak, the 2009 H1/N1 influenza pandemic, the 2013–2016 West African Ebola outbreak, and the COVID-19 pandemic [44]. Since the start of the 21st century, the global economic costs of zoonosis outbreaks have exceeded 100 billion U.S. dollars [45,46]. The current COVID-19 pandemic, affecting millions of people worldwide, has stimulated the development of rapid and sensitive technologies for zoonotic pathogen detection, general patient and health care, and prevention of large-scale outbreaks and further spread, which are of paramount importance in public health [47]. RPA assays are widely used in the detection of zoonotic pathogens because of their high sensitivity, efficiency, expansibility, rapidity, and specificity (Table 2) [48–58].

TABLE 2 | Major zoonotic diseases and RPA detection methods.

Disease	Etiology	RPA method	Amplification time (min)	Temperature (°C)	LOD
Bacterial zoonoses					
Tuberculosis	*Mycobacterium bovis*, *Mycobacterium caprae*, *Mycobacterium microti*	Direct RPA	20	39	6.25 fg
		LF-RPA	25–45	5	5 copies/reaction
		Real-time fluorescence RPA	20	39	4 copies/μl
		Electrochemical RPA	20	39	0.04 ng/μl
		CRISPR/Cas-RPA	180	37	4.48 fmol/L
Brucellosis	*Brucella abortus* *Brucella melitensis*, *Brucella suis*, *Brucella canis*,	Direct RPA	20	38	3 copies/reaction
		LF-RPA	10–30	30–37	6 copies/reaction
		Real-time fluorescence RPA	16	40	17 copies/reaction
Plague	*Yersinia pestis*	CRISPR/Cas-LF-RPA	50	37	10^3–10^6 fg/μl
Leptospirosis	*Leptospira interrogans*	CRISPR/Cas-RPA	60	39	100 copies/ml
Tularemia	*Francisella tularensis*	Real-time fluorescence RPA	20	39–42	10 copies/reaction
		Electrochemical RPA	60	37	500 fM
Lyme disease	*Borrelia burgdorferi*	LF-RPA	30	37	25 copies/reaction
Viral zoonoses					
Rabies	Rabies virus	Direct RPA	20	42	562 copies/reaction
		Real-time fluorescence RPA	15	42	4 copies/reaction
Avian influenza	Influenza A virus	LF-RPA	20	30–42	0.15 pg
		Real-time fluorescence RPA	20	39	100 copies/reaction
Ebola disease	Ebola virus	LF-RPA	40	37	134 copies/μl
Dengue fever	Dengue virus	LF-RPA	23	37	10 copies/μl
		Real-time fluorescence RPA	20	38	14–241 copies/reaction
Zika fever	Zika virus	Real-time fluorescence RPA	20	41	5 copies/reaction
West Nile fever	West Nile virus	Real-time fluorescence RPA	15	39	10 copies/reaction
SARS	SARS coronavirus	LF-RPA	45	42	35.4 copies/μl
		Real-time fluorescence RPA	20	42	7.74 copies/reaction
		CRISPR/Cas-RPA	50	37	1–10 copies/reaction
		Ligation-RPA	30	37	10 copies/reaction
		RPA/rkDNA-graphene oxide probing	96	37	6.0 aM

RPA-LF

Most end-point RPA detection methods reported to date rely on LF assays, and the results can be obtained extremely rapidly in a visual read-out format. LF chromatography test strips are mainly used as simple devices for qualitative and semi-quantitative detection, and can be used in resource-limited or non-laboratory environments [59]. RPA-LF is based on the principle of RPA amplification with biotin-labeled primers and carboxyfluorescein (FAM)-labeled probes for amplification reactions with target nucleic acids; the final amplified product carries both FAM and biotin labels. The detection line of the LF test strip contains streptavidin. When the FAM on the amplicon binds the gold-labeled-anti-FAM antibody in the sample pad, an immune complex is formed. The immune complex undergoes chromatographic diffusion on the strip. The streptavidin on the detection line captures the immune complex containing the biotin amplicon, thus resulting in color development [60]. In addition, multiplex LF strips have been developed, such as the PCRD nucleic acid detector cassette (Abingdon Health, UK), which has two detection lines that can detect FAM/biotin and DIG/biotin labeled amplicons, respectively. This method allows for the detection of various pathogens in the same tube as well as the introduction of an internal control, as has been applied in the detection of three *Anaplasma* species [61].

The limit of detection (LOD) of RPA-LF can be as low as one to ten copies per reaction in the detection of zoonotic pathogens. Wu et al. have established RPA-LF for detecting *Toxoplasma gondii*, with a LOD of 0.1 oocyst/reaction, a value ten times higher than the sensitivity of nested PCR [62]. Shi et al. have used this method to detect avian influenza A virus (H7N9) with a LOD of 32 fg nucleic acid sample, and without cross-reaction with other subtypes of influenza viruses [55]. Rani et al. have presented a rapid, sensitive, specific, and portable method to detect the *rfbE*, *fliC*, and *stx* genes of *Escherichia coli* O157:H7, with LODs as low as 4–5 CFU/mL, 10^1 CFU/mL, and 10^2 CFU/mL, respectively, in 8 minutes at temperatures between 37 and 42°C [53].

RPA-LF has also been used in the detection of other zoonotic parasites, bacteria, and viruses, such as *Trypanosoma cruzi*, *Brucella* spp., *Burkholderia mallei*, *Chlamydia trachomatis*, *Orientia tsutsugamushi*, *Rickettsia typhi*, *Coxiella burnetii*, *Borrelia burgdorferi*, Newcastle disease virus, dengue virus, orf virus, human adenovirus, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [19,20,22,28,49,52,53,57,63–71].

RPA-LF detection can be performed in approximately 20 minutes at 25–45°C. Therefore, simple heating equipment, such as electric water heaters, or even human body heat can be used to achieve accurate detection. The RPA-LF test results show a red band on the strip, which can be observed with the naked eye. Even non-professionals can directly observe the analytic results. This method is highly suitable for on-site detection, particularly in areas with poor economic conditions and limited resources [49,72].

However, LF assays' insufficient accuracy and stability make them unsuitable for quantitative analysis in clinical applications [73]. In addition, during the color development of strips, the lid of the reaction tube must be kept open, thus making the method prone to environmental contamination and false positives Table 3. Many efforts have aimed at addressing this deficiency. Among them, microfluidic technology has shown great advantages, by integrating RT-RPA and a universal LF detection system into a single chip. Results can be obtained with only simple nucleic acid extraction, loading, and incubation for approximately 30 minutes. This MI-IF-RPA detection method is rapid and sensitive, and it effectively decreases the risk of contamination [74].

Real-time fluorescence RPA

As with PCR, RPA amplification results can be monitored by real-time fluorescence [75]. Fluorophore dyes, such as SYBR Green and Eva Green, can be used for real time detection [76,77]. However, these dyes cannot distinguish between amplicons and primer dimers, thus potentially leading to false positive results. Therefore, specific probes are preferred in RPA reactions, including the Exo and Fpg probes, named after the corresponding enzymes [75,78]. The Exo probe carries a fluorescence group and a fluorescence quenching group which are separated by a tetrahydrofuran (THF) residue [79]. During RPA amplification, the DNA repair enzyme exonuclease III cleaves the tetrahydrofuran in the Exo probe, thus leading to separation of the fluorescence group and the fluorescence quenching group, and facilitating the generation of fluorescence that is subsequently monitored.

Generally, real-time fluorescence RPA detection requires less time to complete than RPA-LF. In addition, the lids of the reaction tubes need not be kept open, thereby decreasing the risk of contamination during operation. The only disadvantage of this method is the requirement for thermostatic fluorescence detection instrument, which may limit its ease of use Table 3. The low reaction temperature is advantageous for miniaturization because of the low energy input required; therefore this method is a favorable candidate for battery powered hand-held devices [12]. Researchers have attempted to design and produce simple portable fluorescent readers [80,81].

Ultrasensitive real-time fluorescence RPA methods have been established for the detection of zoonotic pathogens. The LOD for detecting the kinetoplast minicircle DNA of *Leishmania donovani* [80], the CeuE gene of *Campylobacter jejuni* [82], the hipO gene of *Campylobacter coli* [82], and the 18S RNA gene of *Plasmodium knowlesi* [83] has been found to be as low as 1 cell/reaction, 1 CFU/ml, 1 CFU/ml, and 1 plasmid/reaction, respectively.

Euler et al. have developed ten detection methods for eight zoonotic pathogens that are also biothreat agents:

TABLE 3 | Comparison of RPA types.

RPA types	Advantages	Disadvantages
RPA-LF	1. Results can be obtained extremely rapidly with a visual read-out 2. Testing equipment is simple and suitable for resource-limited or non-laboratory environments	1. Insufficient accuracy and stability 2. Proneness to environmental contamination and false positives
Real-time fluorescence RPA	1. Shorter detection time 2. Closed reaction tubes decrease the risk of contamination during operation	Need for a thermostatic fluorescence detection instrument limits ease of use
CRISPR/Cas-RPA	1. The LOD is very low 2. The components of the CRISPR and RPA assays have similar reaction temperatures (37°C), and reactions can be performed in one tube	1. Long detection time 2. Additional labeled ssDNA or ssRNA reporter increases the cost
Electrochemical RPA	Solid phase reaction enables the integration of amplification, hybridization, and detection on a platform, thus decreasing analysis time and contamination	Lower amplification efficiency due to solid phase reaction leads

Francisella tularensis, *Yersinia pestis*, *Bacillus anthracis*, and variola virus, by using RPA assays, and Rift Valley fever virus, Ebola virus, Sudan virus, and Marburg virus by using RT-RPA assays [12]. The analytical sensitivity ranges from 16 to 21 molecules detected, and the detection time ranges from 4 to 10 minutes—a detection performance surpassing those of PCR, real-time PCR, or LAMP [12].

Other zoonotic pathogens for which real-time fluorescence RPA detection has been successfully implemented include *Streptococcus suis* serotype 2, *Mycobacterium tuberculosis*, *Rickettsia* spp., yellow fever virus, dengue virus types 1–4, orf virus, rabies virus, avian influenza virus, hepatitis E virus, Chikungunya virus, Crimean-Congo hemorrhagic fever virus, Zika virus, highly pathogenic porcine reproductive and respiratory syndrome virus, and SARS-CoV-2 [30,48,81,84–94].

Now that various detection methods for single zoonotic pathogens have been established, integrating multiple methods to produce multiplex detection reagents or devices is a major development trend for fluorescence RPA. Researchers have attempted to integrate multiple methods into microfluidic chips to detect dozens of pathogens simultaneously [95,96]. In addition, a mobile suitcase laboratory for detecting zoonotic pathogens in the field and has been used in several studies [80,81,90,97].

CRISPR/Cas-RPA detection

One constraint of isothermal amplification is that single-nucleotide polymorphisms, which are crucial in both pathogen and disease detection, cannot always be discriminated [47,98]. A new molecular diagnostic tool based on the CRISPR/Cas system can overcome this weakness [33]. The origin of CRISPR based detection was rooted in the discovery of the collateral cleavage activity of protein Cas13a. When Cas13a binds CRISPR RNA (crRNA), the crRNA specifically pairs with a target sequence and subsequently induces both enzymatic cleavage of both the targeted sequence and untargeted collateral cleavage of all single stranded RNA (ssRNA) [99]. A comprehensive CRISPR system called SHERLOCK, which combines the CRISPR/Cas system with RPA [33], relies on the collateral trans-cleavage of quenched fluorescent nucleotides after target binding. Over the years, other Cas proteins, including Cas9, Cas12, and Cas14, have also been demonstrated to function in DNA or RNA sensing with high sensitivity and selectivity [47]. Myhrvold et al. have developed a complement to SHERLOCK called HUDSON technology to detect viruses directly from body fluids [100]. Chen et al. have used Cas12a collateral trans-cleavage and isothermal amplification to develop the DETECTR method [101], which can achieve amol/L sensitivity for DNA detection. Li et al. have developed a highly sensitive nucleic acid detection method called HOLMES by using Cas12a and an ssDNA fluorescence probe for rapid detection of DNA and RNA viruses with a sensitivity as low as 1–10 amol/L [102].

Currently, although signal amplification has been improved by introducing spherical nucleic acid reporters or multiple crRNAs [103,104], the sensitivity still does not meet the requirements for clinical detection, and the method is not suitable for application without a nucleotide amplification procedure. To amplify the signal and improve the detection sensitivity, CRISPR/Cas diagnostic technology is usually combined with NAA technology, such as PCR, LAMP, or RPA. Compared with other NAA assays, RPA has an inherent advantage in its compatibility with the CRISPR/Cas system, because both methods use reaction temperatures around 37°C. On the basis of this feature, the All-In-One Dual CRISPR-Cas12a (AIOD-CRISPR) assay for one-pot, ultrasensitive, and visual SARS-CoV-2 detection has been developed [105,106], in which, the components for both RPA and CRISPR-based detection are prepared in one pot, thus avoiding separate pre-amplification of target nucleic acids [101], or physical separation of the Cas enzyme [107].

In the detection of other zoonotic pathogens, to our knowledge, only limited studies have used CRISPR/Cas12a or CRISPR/Cas13a for bacteria or viruses, such as *Leptospira*, *Salmonella* spp., Zika virus, dengue virus, avian influenza A (H7N9) virus, influenza A virus, influenza B virus, and rabies virus [100,108–111].

The limited use of this technology in the detection of zoonotic pathogens may be due to its short duration of application. In addition, compared with RPA-LF and real-time fluorescence RPA, CRISPR/Cas-RPA detection uses additional labeled ssDNA or ssRNA reporter for collateral cleavage, thus potentially increasing the cost Table 3. The advantage of the CRISPR/Cas-RPA detection method in discriminating single-nucleotide polymorphisms may become a disadvantage when mutations are present in the target sequences in clinical applications, which focus on disease diagnosis rather than typing. Thus, this novel and promising detection method requires more studies to support applications in zoonotic pathogen detection.

Electrochemical RPA

Electrochemical RPA detection relies on the rapid isothermal amplification of target pathogen DNA sequences by RPA followed by gold nanoparticle-based electrochemical assessment with differential pulse voltammetry. This method couples RPA and electrochemistry on disposable screen printed carbon electrodes, and electrochemically active substances are used to generate signals associated with NAA [112].

In the detection of zoonotic pathogens, a rapid electrochemical detection method for *Mycobacterium tuberculosis* based on colloidal gold nanoparticles has been reported with a LOD as low as 1 CFU [113]. del Río et al. have constructed an electrochemical platform with a lower LOD of 1×10^{-15} M for *Francisella tularensis* detection [51]. In addition, the electrochemical RPA in detection of SARS-CoV-2 by using human body temperature was established and had a LOD slightly below or comparable to that of RPA assay results obtained from gel electrophoresis without post-amplification purification [114]. The method

usually uses a solid phase and requires a handheld device for electrochemical measurement in on-site detection. The solid-phase RPA approach, on the one hand, allows for integration of DNA amplification, hybridization, and detection on a platform, thus decreasing analysis time and contamination, and potentially enabling on-site testing, but on the other hand, leads to lower amplification efficiency than that in solution, owing to steric hindrance effects on various components in the amplification system Table 3 [115]. However, the electrochemical RPA remains under development, and more work is required to improve its performance and capabilities [115].

Other methods coupled with RPA

Other detection methods coupled with RPA have been reported for zoonotic pathogen detection, including flocculation assays, chemiluminescence, and silicon microring resonator (SMR)-based photonic detection [16].

Flocculation analysis detection is based on the phenomenon of colloidal chemical bridging flocculation. The flocculation assay detection was first combined with an RPA assay reported by Wee et al., in which RPA amplicons on the magnetic bead surfaces cross-link multiple other RPA-magnetic bead conjugates, thus causing a sharp transition from solution phase to flocculate [116], which is detectable by the naked eye. The method was subsequently extended to detect zoonotic pathogens including malaria parasites, *Mycobacterium tuberculosis*, and the influenza virus H1N1 [116,117].

The chemiluminescent detection converts chemical energy into the emission of visible light, as the result of an oxidation or hydrolysis reaction [16]. RPA assays coupled with chemiluminescent detection for several zoonotic pathogens, including HAdV 41, *Legionella* spp. and *Legionella pneumophila*, have been applied on flow-based microarrays [118,119]. This method can be used for multiplex detection after immobilization of one of the two primers from different pathogens on one chip for asymmetric amplification. However, the procedure is tedious and time-consuming, thus limiting its use in field applications and resource-limited regions.

SMR-based photonic detection also involves performing NAA in an asymmetric manner. One primer is pre-immobilized on the SMR, and the binding of nucleic acids to the pre-immobilized primer induces changes in the refractive index proximal to the waveguide surface, which can be monitored in real time through the SMR. Applications in the detection of zoonotic pathogens including *M. tuberculosis* and *F. tularensis*, have demonstrated that SMR-RPA is an alternative detection method to fluorophore-based real-time detection, with the advantages of being label-free and much more sensitive [120,121].

Applications of RPA in SARS-CoV-2 detection

SARS-CoV-2 has become a serious public health concern in recent years [122,123]. Because many infected patients are asymptomatic, the total number of infections remains unclear [124]. The development of sensitive, rapid, specific, and cost-effective detection methods has never been more important. RPA has been widely used for the detection of SARS-CoV-2 viral RNA in clinical samples [71,97,125]. Combined with LF assays, RT-RPA can detect pathogenic nucleic acids within 20 minutes, with a detection limit as low as 7.659 copies/μL RNA [69]. With a microfluidic chip that integrates RT-RPA and universal LF, the detection limit can be increased to 1 copy per/μl, with an incubation time of approximately 30 minutes [74].

The CRISPR-Cas system has recently been used to sensitively detect nucleic acids, and numerous CRISPR-Cas-RPA detection systems have been developed [125–128]. Most of these methods are based on a combination of CRISPR/Cas12a and RT-RPA, with the introduction of a fluorescence probe for fluorescence readout or gold nanoparticles for colorimetric readout [31,122,126,127,129–132]. For example, a CRISPR–Cas12-based assay combined with DNA-modified gold nanoparticles has been developed, with a detection limit of one viral genome sequence copy per test. However, the detection time is increased to 50 minutes, 30 minutes of which are used for colorimetric readings [122,126,127]. Two separate teams have combined CRISPR/Cas9, LF assays, and RT-RPA technology as a platform for visual detection of SARS-CoV-2, thus providing an accurate and convenient pathway for diagnosis of COVID-19 or other infectious diseases in resource-limited regions [128,133]. Few studies have used CRISPR/Cas13a, in which an additional transcription step is needed and may increase the detection time. Arizti-Sanz et al. have identified the optimal conditions to allow single-step Cas13-based detection and RPA, and have developed a sensitive and specific diagnostic tool that can detect SARS-CoV-2 RNA from unextracted samples, with a sample-to-answer time of 50 minutes [134]. Moreover, Tian et al. have designed a system using both Cas12a and Cas13a for dual-gene detection, in which, dual-gene amplified products from the multiplex RPA are simultaneously detected by Cas12a and Cas13a assays in a single tube [135].

In addition to LF assays and the CRISPR-Cas system, many other detection techniques have been combined with RPAs. Wang et al. have established a ligation and recombinase polymerase amplification method. Using a high concentration of T4 DNA ligase, this method has achieved a satisfactory sensitivity of ten copies per reaction within 30 minutes [136]. Furthermore, Moon et al. have combined an rkDNA-graphene oxide probe system with RPA and developed a rapid detection method with extremely high sensitivity (LOD 6.0 aM) [137].

At present, with the continual development of the COVID-19 pandemic, research on rapid and sensitive detection of the SARS-CoV-2 virus remains a development focus.

CONCLUSIONS AND FUTURE PERSPECTIVES

In recent years, zoonoses have caused great economic losses worldwide and severely threatened human health, life, and safety. Because traditional detection technologies can no

longer meet the detection requirements for zoonotic pathogens, rapid, sensitive, specific, multiplex detection methods must be established. As an emerging molecular detection technology, RPA assays have been widely used in medicine and pharmacy applications, and also have begun to emerge in the detection of zoonotic pathogens. RPA assays have many technical advantages: they do not require thermal cycling, and the reaction can be completed at relatively lower temperatures of 37–42 °C; the reaction is fast, with amplification times of 5–20 minutes; the method is portable, and combinations of RPA and LF, fluorescence, the CRISPR/Cas system, and other technologies have been achieved. Since the COVID-19 pandemic, RPA technology has been crucial for rapid pathogen detection.

However, RPA assays are novel, and their use has not been as widespread or common as PCR methods in the detection of zoonotic pathogens, although these methods have developed faster since the COVID-19 pandemic. Because RPA has the advantage of being naturally suitable for on-site testing, more attention is needed in integrating sample preparation with RPA detection to achieve a fast sample-to-result pipeline that would enable a complete RPA assay for on-site or field application. Multiplex high-throughput detection is another research and application direction for zoonotic pathogen detection. In this respect, the combination of microfluid or microarray technology with RPA assay shows good prospects. In addition, as suggested by Li et al. [16], developing wearable sensors and performing RPA assays using human body heat to detect potential zoonotic pathogens may revolutionize RPA diagnostics to enable self-testing. With its continual rapid development, RPA is expected to play a more important role in the prevention and control of zoonotic diseases in the near future, particularly in mobile and point-of-care applications.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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