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Recombinase Polymerase Amplification for Rapid Detection of Zoonotic Pathogens

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Abstract

With the advent of molecular technology, several isothermal techniques for fast detecting 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 implemented in a field-based scenario because the method requires minimal sample preparation and is performed at a constant low temperature (37-42 ℃). It is rapidly becoming a promising tool for rapid detection and further prevention and control of zoonotic diseases. This article will discuss the principles of RPA technology and its derivatives, including RPA coupled with lateral flow test (RPA-LF), real-time fluorescence RPA, Electrochemical RPA, Flocculation RPA and other technologies, and their applications in detection of zoonotic pathogens for a brief review.

Keywords: recombinase polymerase amplification; rapid detection; zoonotic pathogen; zoonosis; RPA

1. Introduction

Nucleic acid amplification (NAA) in vitro, the artificial replication of genetic material, has permeated nearly every field of the life sciences and biotechnology. The development of 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

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rapid detection of nucleic acids and demonstrated to be highly specific and efficient. However, the PCR amplification technique still has several limitations. It relies on the thermal cycler for heating and cooling and high-quality nucleic acids as starting point, which will be limited by the environment and equipment to a certain extent. Furthermore, skilled operators and laboratory environment are also needed, impeding its application in low-resource settings.

In order to overcome the limitations of PCR method, various isothermal DNA amplification methods using various enzymes and amplification systems have been established, including the 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 nucleic acid amplification greatly simplifies the incubation conditions for artificial nucleic acid amplification, and the elimination of thermal cycling reduces the requirements for amplification equipment, making nucleic acids detection no longer dependent on the laboratory environment. Another advantage of the isothermal nucleic acid amplification is that multiple molecular reactions, like denaturation, annealing, and elongation, can be performed asynchronously in one isothermal amplification reaction, which can effectively reduce the nucleic acid amplification reaction time [5, 6]. Among all the isothermal DNA amplification methods, RPA is remarkable due to its simplicity,

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strong specificity and high sensitivity, though with a very short history.

Table 1 Isothermal nucleic acid amplification techniques

Isothermal technique	Template	Primers	Temperature (℃)	Incubation time	Amplified length (bp)	Lyophilised reagents
LAMP	DNA	4~6	60~65	15~60	200	No
RCA	DNA/RNA	1	30~65	60~240	100	No
NASBA	RNA	2	37~42	60~180	100~250	Yes
HDA	DNA	2	60~65	30~120	80~120	No
SDA	DNA	4	30~55	60~120	200	No
RPA	DNA/RNA	2	37~42	20~40	400	Yes

2. Recombinase polymerase amplification (RPA)

RPA was first developed by Niall Armes from ASM Scientific Ltd of Cambridge, UK in 2006 [7]. Unlike the traditional PCR methods, RPA does not rely on thermal denaturation and annealing. Three key enzymes required for the RPA reaction are recombinase that can bind to single-stranded nucleic acid, DNA single-stranded binding protein (SSB), and strand-displacement DNA polymerase[7, 8]. The amplification reaction starts with the combination of the recombinase and a primer with a length of about 30 to 35 nt, the combination complex searches for the target site in double-stranded DNA template. Once the complex is located, it can directly initiate a chain exchange reaction to form a D-shaped loop. SSB then binds to the replaced DNA chain to prevent the primer dissociation. Subsequently, the active hydrolysis of ATP in the recombinase-primer complex leads to a change in the conformation of the complex. After the recombinase disassembled from the nucleoprotein

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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 starts. Both forward and reverse primers enable the amplification reaction to occur in both directions simultaneously. The synthesized amplicon can be used as new template to finally realize an exponential amplification [9].

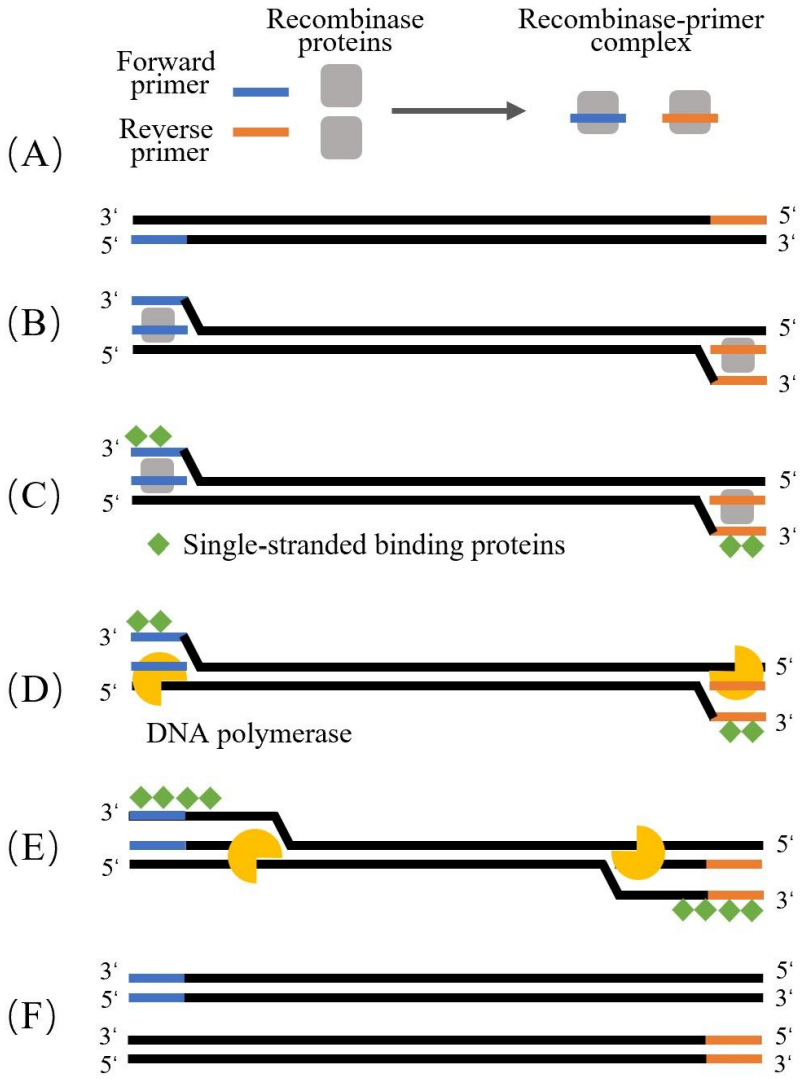

Fig.1. Schematic of RPA principle. (A) The recombinase proteins combine with each primer.

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(B) The combination complex searches for the target site and directly initiate a chain exchange reaction forming a D-loop. (C) Single stranded binding proteins binds to the replaced DNA chain to prevent the primer dissociation. (D) The recombinase disassembled from the nucleoprotein filament and the DNA polymerase extends from the 3' end of primers. (E) DNA amplification reaction starts. (F) Exponential amplification is achieved by cyclically repeating the process.

2.1 Template

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

2.2 Primer and probe

Unlike traditional PCR, the length of RPA primer is generally between 30 and 35 nucleotides, in order to facilitate the formation of a complex between the recombinase and the primer. Longer primers (up to

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45 nucleotides) may produce secondary structure and potential primer artifacts, and lead to less amplification efficiency. Also, there is no melting temperature requirement for RPA primer and probe design. Like primers of traditional PCR, the GC content of the RPA primers should be between 30% and 70%, and long-chain guanines should be avoided at the 5' end, while guanine and cytosine nucleosides can be used at the 3' end to improve performance. The probe is not necessary in the common RPA assay, while is necessary when RPA is combined with various endpoint detection methods addressed below [16-18]. The procedure for primer and probe design is not standardized and no software is available, however, the selected primers and probes can be evaluated using the software for PCR primer design like Primer Premier 5. Usually, multiple groups of primers and probes are designed and evaluated in experiment to screen the best group [19-22] .

2.3 Temperature and incubation time

The optimum reaction temperature of the enzymes used in the RPA assay is between 25 and 42^{o}C, determining the optimum temperature for the RPA reaction is also in this range. RPA assay does not require strict temperature control [23-25]. Even if it exceeds the recommended temperature range, the RPA reaction can still proceed. However, the recommended RPA reaction temperature in most studies is between 37 and 42 ℃ [9, 26].

The time required for nucleic acid amplification to reach a detectable level depends on the concentration of the starting DNA template. At the

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appropriate reaction temperature, it usually takes less than 20 minutes to complete the detection [27]. In practical applications, the amplification results can be observed as low as 3 to 4 minutes. For the solution phase RPA amplification reaction, the recombinase can consume all ATP in the system within 25 minutes. Therefore, too long incubation time is unnecessary. Also, it's recommended to add a step of shaking at the fourth minute of the reaction to improve reaction efficiency [20, 21, 28].

2.4 Advantages and disadvantages of RPA assay

RPA assay offers several advantages. It can carry out nucleic acid amplification under constant temperature conditions of 37-42 ℃ without requiring pre-denaturation steps and high temperature annealing steps. So, RPA does not need expensive thermal cycling equipment, and is suitable for non-instrumented nucleic acid amplification platforms. Researchers have tried to use human body temperature to complete the amplification in various conditions [28].

RPA technology is simple to operate with good expansibility, and can be combined with different detection systems to achieve different detection purposes. Combining with reverse transcriptase makes a reverse transcription-RPA system and can be used to amplify RNA sequences; combining with fluorescence probe makes a real-time fluorescence RPA and can complete a real-time detection; combining with lateral flow (LF) test makes a RPA-LF, and can detect target sequences visually with naked eyes [18, 29-32]. Recently, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated nuclease (Cas) was introduced

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to make a CRISPR-based diagnostic (CRISPR-Dx) named “SHERLOCK” and has promoted the popularity and scope of application of RPA assay [33].

RPA reagents can also be provided in lyophilized form with excellent stability and be transported and stored without refrigeration [34, 35]. As of PCR, multiplex RPA assays that can detect multiple target sequences quickly in one reaction are also available, though depending on the target sequence, amplicon size and primer design [36-38]. All the above advantages facilitate the implementation of RPA in field-based rapid detection applications. In recent years, RPA has been greatly developed in the rapid detection of various pathogens, especially in zoonotic pathogens.

Of course, RPA technology does have some disadvantages. RPA product normally requires purification before agarose gel electrophoresis to avoid smearing caused by other components. Secondly, there is no special software available for the design and screen of RPA primers and probes. It mainly relies on a large number of synthesis and screening work, resulting in increased cost and time consumption. Furthermore, conventional real-time PCR probes (such as Taq-Man probes) are not compatible with the RPA reaction, and the fluorescent fuels are prone to false positive results. In addition, real-time amplification using RPA is not easy to control due to its isothermal amplification properties. Because it is based on a time threshold rather than a cycling threshold, which is dependent on the initial conditions of the reaction, incubation temperature

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and mixing steps. With regard to cost, RPA kits are currently sold by only one company, and the users have limited flexibility in the kit formulations which makes for higher costs when used in small batches.

3. Applications of RPA in zoonotic pathogen detection

Zoonosis, also known as animal-derived disease, refers to any disease or infection that is naturally transmissible from vertebrate animals to humans or from humans to animals [35, 39]. Approximately 60% of emerging human infections are zoonosis in nature, and more than 70% of pathogens originated from wild animal species [40]. At present, there are more than 200 species of zoonotic pathogens known in the world, and the most prevalent ones are anthrax, plague, foot-and-mouth disease, avian influenza, Japanese encephalitis, rabies, etc. [41-43]. Zoonosis is a major public health concern that directly threatens human health. In recent decades, there have been large-scale epidemics of zoonosis, 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 entering the 21st century, the global economic impact of zoonosis outbreaks has exceeded over 100 billion U.S. dollars [45, 46]. The currently ongoing COVID-19 outbreak, affecting millions of people around the globe, again shows the development of rapid and sensitive detection technology for zoonotic pathogens detection, for patient and health care in general, and for risk prevention of large-scale outbreaks and further spread, is paramount

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important in public health [47]. RPA assay is widely used in the detection of zoonotic pathogens due to its high sensitivity, efficiency, expansibility, rapidity, and strong specificity （Table 2） [48-58].

Table 2 Major Zoonotic Diseases and RPA Detection Methods

Disease	Etiology	RPA method	Amplification time (min)	Temperature (℃)	Limit of defection (LOD)
		Bacterial zoonoses			
Tuberculosis	*Mycobacterium bovis*, *Mycobacterium caprae*, *Mycobacterium microti*	Direct RPA	20	39	6.25 fg
		LF-RPA	25~45	5	5 copies/action
		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*	Direct RPA	20	38	3 copies/reaction
	Brucella melitensis,	LF-RPA	10~30	30-37	6 copies/reaction
	Brucella suis,	Real-time	16	40	17 copies/reaction

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	Brucella canis,	fluorescence RPA			
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	20	39	100

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		fluorescence RPA			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

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3.1 RPA-LF

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

The limit of detection (LOD) of RPA-LF can be as low as 1 to 10

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

The RPA-LF is also used in the detection of other various zoonotic parasites, bacteria, rickettsia, and virus, such as *Trypanosoma cruzi*, *Brucella* spp., *Burkholderia mallei*, *Chlamydia trachomatis*, *Orientia tsutsugamushi*, *Rickettsia typhi*, *Coxiella burnetii*, *Borrelia burgdorferi*, Newcastle disease virus (NDV), Dengue virus (DENV) , Orf virus, Human Adenovirus, SARS-CoV-2, et al. [19, 20, 22, 28, 49, 52, 53, 57, 63-71].

RPA-LF detection can be performed around 20 minutes at 25-45^{o}C. Therefore, some simple heating equipment, such as electric water heaters or even body temperature 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 eyes. Even non-professionals can directly observe the analysis results. It is very suitable for on-site detection, especially in areas with poor economic conditions and insufficient resources [49, 72].

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However, LF assays, with insufficient accuracy and stability, are not suitable for quantitative analysis in clinical applications [73]. Also, during the color development procedure of strips, lid of the reaction tube needs to be open, and it is very easy to cause environmental pollution and produce false positive in the following detections. Currently, many efforts have been used to address this deficiency. Among them, microfluidic technology has shown great advantages, it could integrate the RT-RPA and universal lateral flow detection system into a single chip. It only needs simple nucleic acid extraction, loading and incubation for nearly 30min to obtain the results. This MI-IF-RPA detection method is rapid and sensitive and effectively decreases the risk of contamination[74].

3.2 Real-time fluorescence RPA

As of PCR, the results of RPA amplification can be monitored by real-time fluorescence [75]. Fluorophore Dyes, such as SYBR Green and Eva Green can be employed for real time detection[76, 77]. However, these dyes cannot distinguish between amplicons and primer dimers, which can lead to false positive results. Therefore, specific probes are preferred to be used in the RPA reaction, including Exo probe and Fpg probe, named after the enzymes introduced [75, 78]. The Exo probe carries a fluorescence group and a fluorescence quenching group, which are respectively combined with a thymine, separated by a tetrahydrofuran (THF) base [79]. During the process of RPA amplification, the DNA repair enzyme Exonuclease III will cut off the tetrahydrofuran (THF) in the Exo-probe, leading to the separation of the fluorescence group and the

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fluorescence quenching group, and thus facilitating the generation of fluorescence to be monitored.

Generally, real-time fluorescence RPA takes less time to complete the detection than RPA-LF. Also, the lids of the reaction tubes do not need to be open, which reduces the risk of contamination during operations. The only disadvantage of this method is that a thermostatic fluorescence detection instrument is needed, which may limit its ease of use. The low reaction temperature shows an advantage for miniaturization since it needs much less energy input and is therefore better candidate for battery driven hand-held devices [12]. So, researchers have tried to design and produce simple portable fluorescent reader [80, 81].

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

Based on the real-time fluorescence RPA, Milena Euler et al. developed 10 detection methods towards 8 zoonotic pathogens, which are also biothreat agents, including *Francisella tularensis*, *Yersinia pestis*, *Bacillus anthracis*, and variola virus using RPA assays, and Rift Valley fever virus, Ebola virus, Sudan virus, and Marburg virus using reverse transcriptase RPA (RT-RPA) assays [12]. Their analytical sensitivities ranged from 16 to 21 molecules detected and the detection time ranged

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from 4 to 10 min, which showed a detection performance better to PCR, real-time PCR or LAMP [12].

In addition, other zoonotic pathogens, for which the real-time fluorescence RPA detection method has successfully implemented, include *Streptococcus suis* serotype 2 (SS2), *Mycobacterium tuberculoss*, *Rickettsia* spp.,Yellow fever virus, dengue virus types 1-4, Orf virus, Rabies virus, Avian influenza virus, Hepatitis E virus (HEV), Chikungunya virus (CHIKV), Crimean-Congo Hemorrhagic fever virus (CCHFV), Zika virus, Highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV), SARS-CoV-2, etc [30, 48, 81, 84-94].

Nowadays, since various detection methods towards single zoonotic pathogen have been established, integrating multiple methods to make multiplex detection reagents or devices is one of the development trends of fluorescence RPA method. Researchers have tried to integrate multiple methods into microfluidic chip to detect dozens of pathogens simultaneously [95, 96]. Also, when detecting zoonotic pathogens in the field, a mobile suitcase laboratory is necessary and has been applied in several studies [80, 81, 90, 97].

3.3 CRISPR/Cas-RPA detection

One of the constraints of isothermal amplification is that single-nucleotide polymorphisms (SNPs), which are crucial in both pathogen and disease detection, cannot always be discriminated [47, 98]. Recently a new molecular diagnostic tool based on CRISPR/Cas system

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has been developed and can overcome this weakness [33]. The origin of CRISPR based detection lies in the discovery of the collateral cleavage activity of protein Cas13a. When Cas13a is associated with a CRISPR RNA (crRNA), this crRNA can specifically complement with a target sequence, inducing enzymatic cleavage of both the targeted sequence and untargeted collateral cleavage of all single stranded RNA (ssRNA)[99]. Then a comprehensive and applicable CRISPR system named SHERLOCK, which combined the CRISPR/Cas system with RPA, was developed [33], relying on the collateral trans-cleavage of quenched fluorescent nucleotides after target binding. Over the years, other Cas proteins, including Cas9, Cas12, and Cas14, has also proven to work for DNA or RNA sensing with high sensitivity and selectivity [47]. Myhrvold et al. developed "SHERLOCK" as “HUDSON” technology to detect viruses directly from body fluids [100]. Chen et al. used Cas12a collateral trans-cleavage and isothermal amplification to develop the "DETECTR" method [101], which achieved amol/L sensitivity for DNA detection. Li et al. developed a highly sensitive nucleic acid detection method "HOLMES" using Cas12a and ssDNA fluorescence probe, which is used for rapid detection of DNA and RNA viruses with a sensitivity as low as 1-10 amol/L [102].

Nowadays, though signal amplification has been improved by introducing spherical nucleic acid (SNA) reporter or multiple crRNA [103, 104], the sensitivity still cannot meet the requirement of clinical detection and is not suitable for application without a nucleotide

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amplification procedure. In order to amplify the signal and improve the detection sensitivity, CRISPR/Cas diagnostic technology is usually combined with NAA technology, such as PCR, LAMP, and RPA. Compared with other NAA assays, RPA has inherent advantage in cooperating with CRISPR/Cas system, because they share the similar reaction temperature around 37^{o}C. Based on this feature, 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 were prepared in one-pot, completely circumventing the separate pre-amplification of target nucleic acids [101], or physical separation of Cas enzyme [107].

In detection of other zoonotic pathogens, to our knowledge, only limited studies have been conducted based on CRISPR/Cas12a or CRISPR/Cas13a, towards bacteria or viruses like *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 detection zoonotic pathogens may account for the short development time. Also, compared with RPA-LF and real-time fluorescence RPA, CRISPR/Cas-RPA detection uses extra labelled ssDNA or ssRNA reporter for collateral cleavage, which may increase the cost. The advantage of the CRISPR/Cas-RPA detection method in discriminating SNPs may become a disadvantage when there is a mutation in the target sequence in clinical application,

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which focuses on disease diagnosis rather than typing. Anyway, as a novel and promising detection method, more studies are needed in application of zoonotic pathogen detection.

3.4 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. It couples RPA and electrochemistry on disposable screen printed carbon electrodes, and electrochemically active substances are used to generate signals related to nucleic acid amplification [112]. and is capable making the.

In detection of zoonotic pathogens, a rapid electrochemical detection method for *Mycobacterium tuberculosis* based on colloidal gold nanoparticles has been reported with LOD as low as 1 CFU [113]. Jonathan Sabaté del Río et al. constructed an electrochemical platform with a lowered LOD of 1×10^{-15} M and conducted on *Francisella tularensis* detection [51]. Also, the electrochemical RPA in detection of SARS-CoV-2 using human body temperature was established and performed LOD slightly lower than or comparable to that of RPA assay results obtained by gel electrophoresis without post-amplification purification [114]. The method usually uses a solid phase and needs a handheld device for electrochemical measurement in the on-site detection. The solid-phase RPA approach, on one hand, allows integrating DNA amplification, hybridisation and detection on a platform, thus reducing

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analysis time and contamination, which paves the road to on-site testing, on the other hand, leads to lower amplification efficiency than in solution due to the steric hindrance effect on various components in the amplification system [115]. However, the electrochemical RPA is still incipient, and more work is required for improving its performance and capabilities [115].

3.5 Other methods coupled with RPA

Some other different detection methods coupled with RPA, have been reported to be applied in zoonotic pathogen detection, including flocculation assay, chemiluminescent, and silicon microring resonator (SMR)-based photonic [16].

Flocculation analysis detection is based on the phenomenon of colloidal chemical bridging flocculation. The flocculation assay detection was first combined with RPA assay by Wee EJ, in which, in positive results, RPA amplicons on the magnetic bead surfaces cross-linked multiple other RPA-magnetic bead conjugates, causing a sharp transition from solution phase to flocculate[116]. The transition can be judged by naked eyes. The method was subsequently extended to detect zoonotic pathogens like malaria parasites, *Mycobacterium tuberculosis*, and 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]. The RPA assay coupled with chemiluminescent detection method for some zoonotic pathogens, including HAdV 41, *Legionella* spp.

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and *Legionella pneumophila* have been applied on flow-based microarrays [118, 119]. It's suitable for multiplex detection by immobilizing one of the two primers from different pathogens on one chip for asymmetric amplification. However, the procedure is tedious and a little time-consuming, which may limit its use in the field and source-limited regions.

As of the chemiluminescent detection, SMR-based photonic detection also involves performing nucleic acid amplification in an asymmetric manner. One of the primers is pre-immobilized on the SMR, and the binding of nucleic acids to pre-immobilized primers will induce changes in the refractive index proximal to the waveguide surface, which can be monitored in real-time on the SMR. The applications on detection of zoonotic pathogens including *M. tuberculosis* and *F. tularensis*, have proven the SMR-RPA detection is an alternative detection method for fluorophore-based real-time detection, yet is label-free and much more sensitive [120, 121].

Table 3 Comparetion of each RPA types

RPA types	Advantages	Disadvantages
RPA-LF	1. Results can be obtained extremely rapidly in a visual read-out format; 2. Testing equipment is simple and suitable for resource-limited or non-laboratory environments.	1. Insufficient accuracy and stability; 2. It is very easy to cause environmental pollution and produce false positive.
Real-time fluorescence RPA	1. Shorter detection time; 2. Closed reaction tubes reduces the risk of contamination during operations;	The thermostatic fluorescence detection instrument limits its ease of use.

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CRISPR/Cas-RPA	1. The limit of detection (LOD) is very low; 2.The components of the CRISPR and RPA assays have similar reaction temperature(37 ℃), and can be reacted in one tube..	1. Long detection time; 2. Extra labelled ssDNA or ssRNA reporter increases the cost.
Electrochemical RPA	Solid phase reaction allows integrating amplification, hybridisation and detection on a platform, reducing analysis time and contamination.	Solid phase reaction leads to lower amplification efficiency

3.6 Applications of RPA in SARS-CoV-2 detection

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has become a serious public health concern in recent years[122, 123]. As there are numerous asymptomatic infected patients, 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]. Combine with the lateral flow assays, reverse transcriptase recombinase polymerase amplification can complete the detection of pathogen nucleic acids within 20min and the detection limit can be as low as 7.659 copies/μL RNA[69]. Utilizing a microfluidic chip that integrates RT-RPA and universal lateral flow, the

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detection limit can be increased to 1 copy per/μl, with an incubation time of approximately 30 minutes[74].

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 studies are based on combination of CRISPR/Cas12a and RT-RPA, with introduction of fluorescence probe for fluorescence readout or gold nanoparticles (AuNPs) for colorimetric readout [31, 122, 126, 127, 129-132].For example, the technique of the CRISPR–Cas12-based assay combine with the DNA-modified gold nanoparticles (AuNPs) has been developed, making the detection limit to 1 copy of viral genome sequence per test. But the detection time has been increased to 50 minutes, of which 30 minutes were used for colorimetric readings[122, 126, 127]. Two separate scientific teams combined CRISPR/Cas9, LF assay, and RT-RPA technology as a platform for visual detection of SARS-CoV-2, providing an accurate and convenient pathway for diagnosis of COVID-19 or other infectious diseases in resource-limited regions [128, 133]. Few studies are based on CRISPR/Cas13a, in which, an extra transcription step is needed

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and may elongate the detection time. Arizti-Sanz J et al. identified the optimal conditions to allow Cas13-based detection and RPA to occur in a single step and 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 min [134]. Moreover, Tian T. et al. designed a system using both Cas12a and Cas13a for dual-gene detection, in which, dual-gene amplified products from the multiplex RPA were simultaneously detected by Cas12a and Cas13a assay in a single tube [135].

In addition to LF assay and CRISPR-Cas system, many other detection techniques have been combined with the RPA. Pei Wang et al. established a ligation and recombinase polymerase amplification method (L/RPA). Using the high concentration of T4 DNA ligase, this method achieved a satisfactory sensitivity of 10 copies per reaction within 30 min[136]. Furthermore, Moon H.C. et al. combined an rkDNA-graphene oxide (GO) probe system with RPA and developed a rapid detection method exhibiting extremely high sensitivity (LOD 6.0 aM)[137].

At present, with the continuous development of the SARS-CoV-2

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epidemic, research on rapid and sensitive detection of SARS-CoV-2 virus is still a development focus.

4. Conclusions and future perspectives

In recent years, the existence and prevalence of zoonosis in the world have caused great economic losses, and also seriously threatened the health and life safety of the people. Traditional detection technologies can no longer meet the detection requirements of zoonotic pathogens, so it is necessary to establish rapid, sensitive, specific, and multiplex detection methods. As an emerging molecular detection technology, RPA assay has not only been widely used in medicine and pharmacy, but also has begun to emerge in the detection of zoonotic pathogens. RPA assay has many technical advantages: it does not require thermal cycling, and the reaction can be completed at lower temperature of 37 to 42 ℃; the reaction is fast with amplification time from 5 to 20 min; it is convenient to carry, and the combination of RPA and LF, fluorescence, CRISPR/Cas system, and other technologies have been realized. Since the pandemic of SARS-CoV-2, RPA technology has performed crucial role in rapid detection of the pathogen.

However, RPA assay is novel and has not been as widespread or popular as PCR method in detection of zoonotic pathogens, though it has developed faster since the pandemic of SARS-CoV-2. Considering that RPA has the advantage of being naturally suitable for on-site test, more attention is needed for integrating sample preparation with the RPA

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detection, resulting in fast “sample-to-result”, which would largely facilitate a complete RPA assay for on-site or field application. Multiplex and high-throughput detection is another research and application direction for zoonotic pathogen detection, and in this respect, combination of microfluid or microarray technology with RPA assay is a good prospect. Also, as the viewpoint of Jia Li et al. [16], developing wearable sensors and performing a fast RPA assay using people’s body temperature to detect potential zoonotic pathogens could revolutionize RPA diagnostics to be a self-testing. Anyway, with its continuous fast development, it is believed that RPA will play a more important role in the prevention and control of zoonotic diseases in the near future, especially in mobile and point-of-care applications.

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