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REVIEW

Isothermal amplification-based assays for rapid and sensitive detection of severe acute respiratory syndrome coronavirus 2: Opportunities and recent developments

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Summary

The coronavirus disease 2019 (COVID-19) is a global pandemic caused by a novel coronavirus called severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). To date, the virus has been detected in 219 countries of the world. Therefore, managing the disease becomes the priority, in which detecting the presence of the virus is a crucial step. Presently, real-time RT polymerase chain reaction (RT-qPCR) is considered a gold standard nucleic acid amplification test (NAAT). The test protocol of RT-qPCR is complicated, places high demands on equipment, testing reagents, research personnel skills and is expensive. Therefore, simpler point-of-care (POC) tests are needed to accelerate clinical decision-making and take some of the workload from centralized test laboratories. Various isothermal amplification-based assays have been developed for the sensitive detection of different microorganisms, and recently some of them have been applied for detection of SARS-CoV-2. These do not require any programable thermocycler, can produce the results in a single temperature, and therefore, are considered simple. Unlike RT-qPCR, these methods are highly sensitive, specific, less time-consuming, simple and affordable, and can be used as POC diagnostic kit for COVID-19. In this review, we have discussed the potential of isothermal amplification-based assays as an alternative to RT-qPCR for the detection of SARS-CoV-2.

KEYWORDS
isothermal amplification-based assays, molecular diagnostics, novel coronavirus, rapid detection, SARS-CoV-2

1 | INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel coronavirus, causes coronavirus disease 2019 (COVID-19). After it affected over 110 countries, WHO declared this disease a global pandemic on 11 March 2020. A significant number of infected individuals are asymptomatic, and the number of infected individuals in many countries may be underestimated due to limited testing

Abbreviations: AMV, avian myeloblastosis virus; CDC, Centres for Disease Control and Prevention; COVID-19, coronavirus disease 2019; HAD, helicase-dependent amplification; HSV, herpes simplex virus; LAMP, loop-mediated isothermal amplification; LOD, limit of detection; NAAT, nucleic acid amplification test; NASBA, nucleic acid sequence-based amplification; PEDV, porcine epidemic diarrhoea virus; POC, point-of-care; PSR, polymerase spiral reaction; RCA, rolling circle amplification; RPA, recombinase polymerase amplification; RT-LAMP, reverse transcriptase LAMP; RT-qPCR, real-time RT polymerase chain reaction; RT-tHDA, reverse transcription-thermophilic HDA; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SSB, single-strand binding.

capacity. The availability of sophisticated testing facility in many districts of India is a serious limitation. Even in some of the developed countries, testing is limited to only symptomatic individuals. Hence asymptomatic carriers are not detected, and they could continue to spread the virus. To estimate the actual magnitude of the disease, a rapid and affordable diagnostic method is essential.

Usually, the detection of RNA virus (causing acute respiratory infection, in particular) is carried out by probe-based real-time RT polymerase chain reaction (RT-qPCR).[1–7] The method has been well accepted, as it has a high level of sensitivity and can offer very low limit of detection (LOD). However, performing RT-qPCR requires sophisticated instruments, long reaction times, and due to the complexity associated with result analysis, skilled personnel are required. Furthermore, false-negative results at a late stage of the disease due to low viral load in the upper respiratory tract could be a problem. On the other hand, the serology tests for COVID-19 have also been recommended by the CDC, but this is an indirect method of detecting SARS-CoV-2, as it detects antibodies in patients' blood.[8] Hence, alternative rapid, portable, and affordable nucleic acid amplification tests (NAAT) are required for the detection of SARS-CoV-2.

Unlike PCR-based methods, isothermal amplification-based assays are simple processes that rapidly amplify the nucleic acids at a constant temperature and do not require any programmable thermocycler.[9] These methods are well-accepted since they are highly sensitive, specific and simple over other molecular diagnostic techniques. During amplification, high pyrophosphate ion by-product levels are produced, which can change the pH of the reaction mixture. Therefore, a pH-sensitive dye can be used for simple visual detection.[10] Various isothermal amplification methods that can be used for the detection of SARS-CoV-2 are listed in Table 1. Looking at these assays' potential, regulatory authority like US Food and Drug Administration has approved the use of kits based on isothermal nucleic acid amplification (https://www.fda.gov/media/138248/download). Here we have reviewed the potential applications of isothermal amplification assays for the detection of SARS-CoV-2 to combat COVID-19.

2 | ADVANTAGES AND DISADVANTAGES OF ISOTHERMAL NUCLEIC ACID AMPLIFICATION ASSAYS

While various isothermal assays have been developed and used for nucleic acid amplification, loop-mediated isothermal amplification (LAMP) assay is relatively popular, developed by Notomi et al.[11] The isothermal amplification-based assays involve simple pre and post-processing methods. The sample collection to completion of the test requires very little time compared to other molecular methods like qPCR. Therefore, these have the potential of a point-of-care (POC) diagnostic tool for detection of SARS-CoV-2. One of the important pre-requisites in any molecular diagnostic technique is the use of purified target nucleic acid. It is generally time-consuming and cost-intensive to obtain a relatively pure form of nucleic acid. However, in isothermal amplification-based assays, this pre-requisite can be done away with since crude cell lysate can be used as a template for the reaction. Moreover, the end products can be detected in various ways, such as visualization by the naked eye, using a turbidimeter, lateral flow dipstick, or agarose gel electrophoresis. On the other hand, these isothermal amplification-based assays usually provide only qualitative results and can be susceptible to contaminations and chances of generating false-positive results.

3 | DETECTION OF SARS-CoV-2 BY ISOTHERMAL AMPLIFICATION-BASED ASSAYS

Some of the isothermal amplification-based assays have been developed for the sensitive and accurate detection of SARS-CoV-2 (Table 2). An overall detection process of SARS-CoV-2 using different isothermal amplification-based assays was shown in Figure 1. However, few of these methods have been optimised for the detection of a novel coronavirus, SARS-CoV-2 using specific genes such as *RdRp*, *spike*, *E* and *nucleocapsid* genes (Figure 2). The updates on these methods for the detection of SARS-CoV-2 have been discussed here.

3.1 | Loop-mediated isothermal amplification

LAMP assay is one of the most studied isothermal amplification assays and has been used to detect various microorganisms. Various investigators across the globe have been working on developing LAMP-based POC diagnostic tool for the detection of SARS-CoV-2. This method uses a single temperature for the amplification of nucleic acid. The method requires *Bst* polymerase enzyme with strand displacement and polymerization activity and four primers that bind six regions in the target during the amplification.[11] RT-LAMP assay is a variant of the conventional LAMP assay, uses RNA as a template, RT enzyme for the synthesis of cDNA, *Bst* polymerase and primers. Initially, the RT-LAMP was developed for the detection of West Nile Virus by Parida et al.[12] The RT-LAMP has been used as a diagnostic tool for different viruses, including SARS-CoV-2. Target genes for the detection of SARS-CoV-2 include *orf1ab*, *nucleocapsid phosphoprotein* (*N*), *spike protein* gene (*S*), *envelope protein* (*E*) gene, membrane glycoprotein gene.[13–17] Baek et al.[18] reported RT-LAMP as a rapid early-detection method for novel SARS-CoV-2, and the assay allows the detection of SARS-CoV-2 in 30 min with the detection limit of 10^2 RNA copies. A CRISPR–Cas12 combined LAMP assay was developed and found to be faster as compared to the Centres for Disease Control and Prevention (CDC) recommended real-time RT–PCR assay. The assay also showed 95% positive predictive agreement and 100% negative predictive agreement.[19] Zhang et al.[20] developed RT-LAMP assay targeting five different regions of the virus, which can be performed using direct tissue samples or cell lysate. This finding is very important because nucleic acid extraction usually consumes significantly more time and also increases the cost

TABLE 1 Comparison between various isothermal amplification-based assays

Characteristics	Name of the assay					
	RT-Loop mediated isothermal amplification	Nucleic acid sequence-based amplification	RT-Polymerase spiral reaction	Helicase-dependent amplification	Recombinase polymerase amplification	Rolling circle amplification
Recommended enzyme[a]	*Bst* polymerase, RT	AMV RT, RNase H and T7 DNA dependent RNA polymerase	*Bst* polymerase, reverse transcriptase	DNA polymerase enzyme and helicase enzyme	Recombinase enzyme and strand-displacing polymerase	phi29 or *Bst* polymerase
Nucleic acid target	DNA or RNA	Generally RNA	DNA or RNA	DNA or RNA	DNA or RNA	DNA or RNA
Temperature required	60°C–65°C	41°C	60°C–65°C	60°C–65°C	37°C–45°C	30°C–37°C
Estimated reaction time or assay time[b]	30–60 min	1–2 h	30–60 min	1–2 h	30 min	30 min
Effect of inhibitors to the assay[c]	Not observed	Observed	Not observed	Not observed	Observed	Observed
Developed for the detection of SARS-CoV-2[d]	Yes	No	Yes	No	Yes	No
Instrument used for the assay[e]	Simple heat block	Simple heat block	Simple heat block	Simple heat block	Simple heat block	Simple heat block
Space taken by the instrument[f]	Minimum	Minimum	Minimum	Minimum	Minimum	Minimum
Sensitivity and specificity[g]	98%–100% and 75%–100%	No data	97%–100% and 65-100%	No data	86%–99% and 65%–100%	No data
Approximate cost per test[h]	$4–$8/reaction	No data	$4–$8/reaction	No data	$8–$15/reaction	No data
Ability to test in rural areas	Yes	Yes	Yes	Yes	Yes	Yes
Ability for door to door testing	Yes	Yes	Yes	Yes	Yes	Yes
Ability to do multiple samples at the time	Yes	Yes	Yes	Yes	Yes	Yes
Generation of biomedical waste[i]	Minimum	Minimum	Minimum	Minimum	Minimum	Minimum

[a]Alternative enzymes can be used for the assay.

[b]Excluding the DNA or RNA extraction time.

[c]May depends on the optimization of the assay.

[d]Based on the information available to date.

[e]In-general, non-programmable heat block such as dry bath incubator or any other simple. incubator is required. However, relatively sophisticated instrument such as thermocycler can be used.

[f]Can be carried out in a very small space.

[g]Based on the information available to date for severe acute respiratory syndrome coronavirus 2 detection.

[h]Just a rough estimation, the actual cost will vary due to various factors.

[i]Minimum as compared to real-time reverse transcriptase polymerase chain reaction.

of the test. In a comparison study conducted by Huang et al.[21] between the gold standard method, RT-qPCR assay and RT-LAMP, similar types of results and statistically good agreement was demonstrated. They also found that RT-LAMP could show positive amplification for the sample which had a Ct value of 36 in RT-qPCR. When swab samples are used in the validation of RT-LAMP assay, they found that when the viral load is high RT-LAMP assay is suitable to detect the infection but if there is low viral load sensitivity of RT-LAMP assay is not enough to detect SARS-CoV-2.[22] COVID-19 pandemic has shown us the importance of POC diagnostic tool in controlling the pandemic; hence RT-LAMP assay can be used in the field of diagnosis for the detection of SARS-CoV-2.

TABLE 2 Comparison of isothermal amplification-based assays with other presently available methods for the detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)

Name of diagnostic method	Real-time reverse transcriptase polymerase chain reaction	Serological tests[a]	Computed tomography	Isothermal amplification-based assays[b]
Sensitivity	High	Medium	Low	High
Specificity	High	Medium	Low	High
Technical complexity	High	Simple	High	Low
Quantitative detection possible	Yes	No	No	Yes
Consideration as point-of-care diagnostic	No	Yes	No	Yes
Requirement of skill personnel	Yes	No	Yes	Yes/No
Requirement of sophisticated equipments	Yes	No	Yes	No
Overall time involved	Relatively rapid	Very rapid	Relatively rapid	Rapid
Cost-effectiveness	Expensive	Less expensive	Expensive	Less expensive
Capability for the detection of SARS-Cov-2	Very high	High	Medium	Very high

[a]Both antigen and antibody-based tests.

[b]Includes all types of isothermal amplification-based assays.

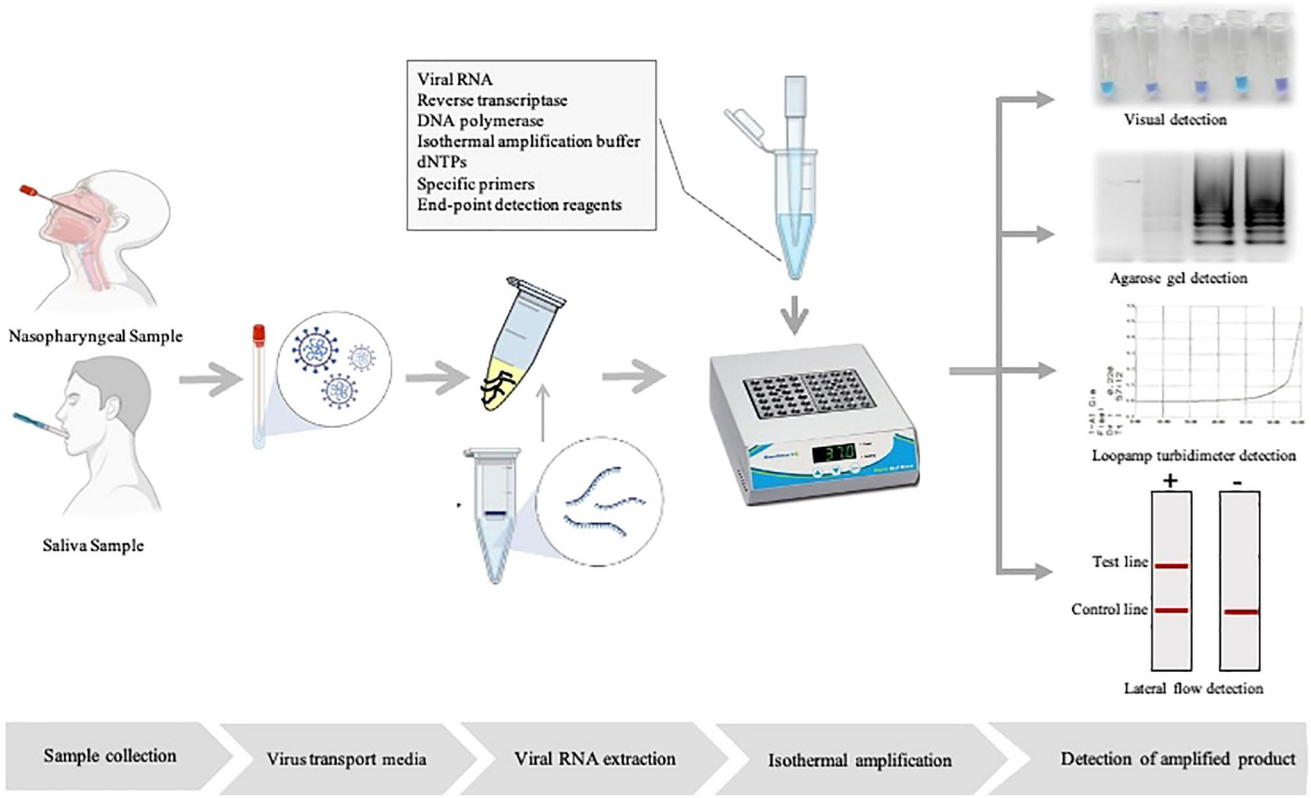
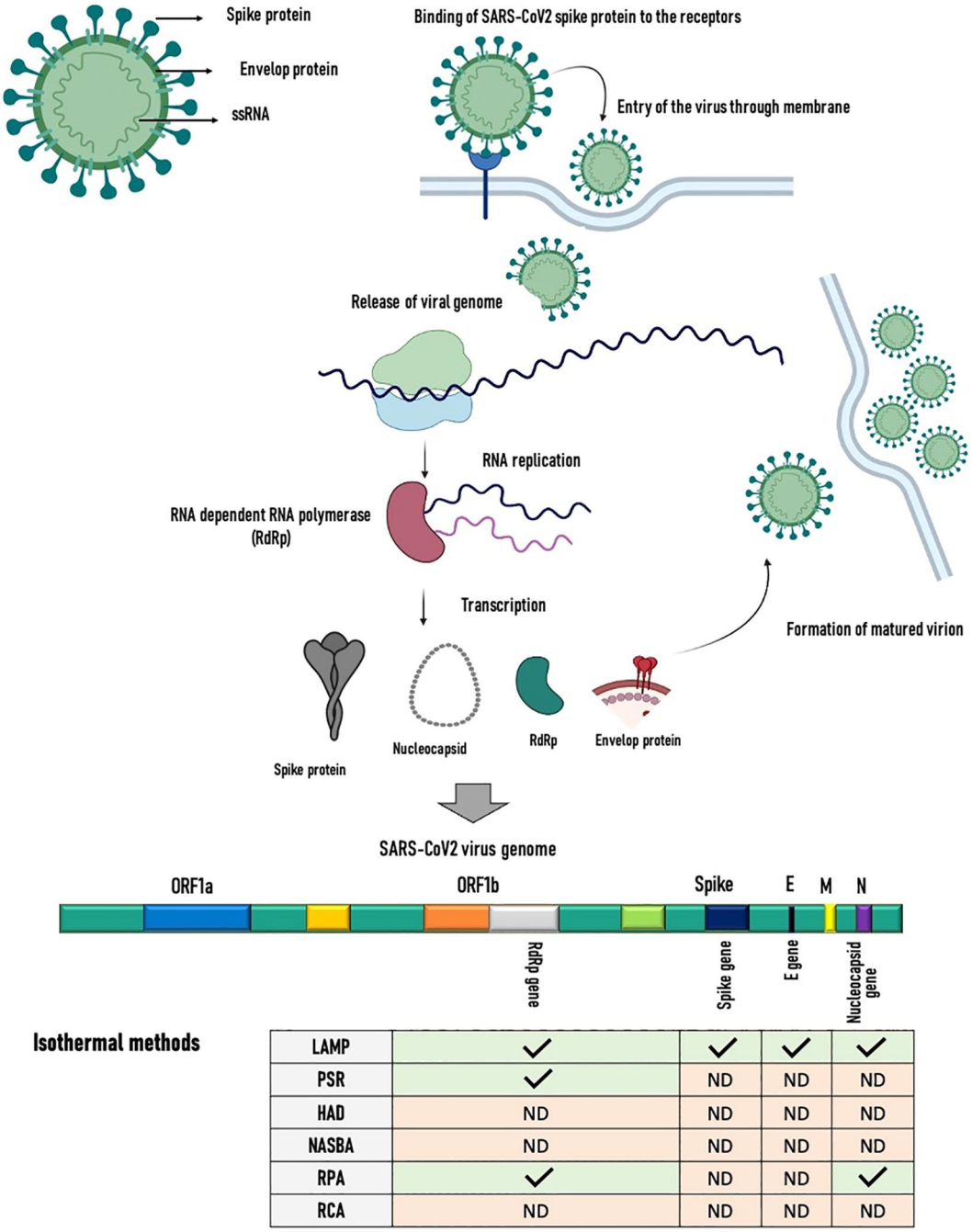


FIGURE 1 Detection of severe acute respiratory syndrome coronavirus 2 using isothermal amplification assays

3.2 | Nucleic acid sequence-based amplification

The nucleic acid sequence-based amplification (NASBA) is an isothermal transcriptional based nucleic acid amplification assay, which is also known as 'self-sustained sequence replication' (3SR).[23] The NASBA is majorly used for the in vitro amplification of RNA in a short time. The assay requires two sets of primers and three different enzymes, including avian myeloblastosis virus (AMV) RT, RNase H and T7 DNA dependent RNA polymerase.[24,25] The assay can generate 10^{13} copies from an initial copy number of 10^{4} in 2 hours.[23] Lu et al.[26]



Isothermal methods	RdRp gene	Spike gene	E gene	Nucleocapsid gene
LAMP	✓	✓	✓	✓
PSR	✓	ND	ND	ND
HAD	ND	ND	ND	ND
NASBA	ND	ND	ND	ND
RPA	✓	ND	ND	✓
RCA	ND	ND	ND	ND

FIGURE 2 Different isothermal methods have been developed targeting various regions (genes) of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). '✓' indicates studies carried out. ND: No data

developed colorimetric split G-quadruplex DNAzyme assay with NASBA assay to detect the classic swine fever virus. NASBA-ELISA has developed grass carp reovirus and found it to be 100% specific and showed the sensitivity of 14 copies/μL within 5 h. The assay NASBA has experimented for the detection of HCV, rhinovirus, human papillomavirus, human cytomegalovirus in HIV-1, hepatitis A.[27–31] Real-time NASBA test for SARS-CoV was developed by Keightley et al.[32] The study compared the RT-NASBA and RT-PCR and found that both the methods were equally sensitive in the detection of SARS-CoV. Studies have been conducted using NASBA assay to detect microorganisms having RNA as genetic material because this method can synthesise only ssRNA and ssDNA.[33] Even though the assay offers good sensitivity, it has few drawbacks, like it can be used only for single-stranded genetic material. The assay requires multiple enzymes for the amplification that can increase the test cost. However, considering the sensitivity and specificity of the assay, it can be a good option for the detection of RNA-based viruses and has the potential to be used as POC diagnostics for the detection of SARS-CoV-2.

3.3 | Polymerase spiral reaction

Polymerase spiral reaction (PSR) is a simple assay and can be performed in a single temperature. Like PCR assay, the PSR utilises two primers that would bind to the target gene and amplify the target DNA. The PSR assay majorly requires a heat-resistant strand displacement DNA polymerase like *Bst* polymerase enzyme and one pair of primers consisting region for the amplification and spiral formation. Additionally, an extra pair of primers can be used to boost the amplification. To date, PSR assay has been used for a limited number of studies for the detection of viruses and bacteria. Liu et al.[34] developed PSR assay for the first time targeting bla_{NDM-1} in *Escherichia coli* and reported it to be a rapid, highly sensitive, and specific method along with detection limit of 6 CFU/reaction. Later, Dong et al.[35] developed PSR assay for the detection of *Pseudomonas aeruginosa* targeting *toxR* gene. PSR was developed for the detection of bacteria such as *Salmonella* and *Vibrio parahaemolyticus*.[36,37] For the detection of viruses, Gupta et al.[38] developed PSR assay for the detection of canine parvovirus 2 and reported that the assay is more sensitive as compared to conventional PCR. RT-PSR assay was developed for the detection of RNA viruses like porcine epidemic diarrhoea virus (PEDV).[39] In another study by He et al.,[40] RT-PSR was found to be 10 times more sensitive than the RT-qPCR for the detection of coxsackievirus. Recently Tran et al.[41] have developed PSR along with another isothermal assay that is, colorimetric isothermal NAATs, for the detection of SARS-CoV-2 targeting *N* gene and *OFR1ab* region. These studies suggested that PSR can be developed as a good alternative to other nucleic acid-based diagnostic methods for sensitive and rapid isothermal for the detection of microorganisms. Therefore, further studies can be carried out on the PSR-based detection of SARS-CoV-2 as an alternative assay.

3.4 | Helicase-dependent amplification

Helicase-dependent amplification (HDA) is an isothermal amplification method that does not require thermal variation for detachment double-stranded nucleic acid and polymerization. In HDA, the unwinding of dsDNA is carried out by helicase enzyme and polymerization by DNA polymerase enzyme, which lacks exonuclease activity. In HDA, another important component used is single-strand binding (SSB) protein, which is involved in the prevention of reannealing and degradation of ssDNA.[42,43] Using HAD method, several viruses and bacterial detection have been performed. Goldmeyer et al.[44] developed one-tube isothermal reverse transcription-thermophilic HDA (RT-tHDA) for the detection of RNA viruses such as enterovirus and ebola virus. The study showed that RT-tHDA could detect as low as 0.2 pg of total human RNA and could able to produce millionfold amplification just in 10 minutes. There are other studies where investigators have developed HAD for the detection of herpes simplex virus (HSV) and HIV-1 etc.[45,46] Although there have been studies that used HAD method for the detection of RNA viruses, no study on RT-HDA has been reported so far for the detection of SARS-CoV-2. The method can be considered and optimized for the sensitive detection of SARS-CoV-2.

3.5 | Recombinase polymerase amplification

The recombinase polymerase amplification (RPA) was discovered in 2006 by Piepenburg et al.[47] It is a relatively simple isothermal assay that can be performed in a short period of time.[48,49] In this method, recombinase enzyme plays an important role, hence the name RPA. Initially, recombinase enzyme forms a complex with primers and unwind the duplex DNA, later DNA polymerase enzyme displaces the strands and amplifies the nucleic acid.[43,50] The nucleic acid-based technique has been carried out for the detection of various microorganisms. RPA assay for the detection of HIV-1 was developed as a rapid detection method without the use of any heating instrument, optimized in ambient temperature.[48] Huang et al.[51] developed RPA-CRISPER based detection method for the sensitive detection of SARS-CoV-2. The high-throughput CRISPR-p based RPA assay was able to deliver the results in approximately 50 min, and the detection limit was two copies per sample. This method has lot of potential to be a diagnostic tool for the detection of SARS-CoV-2. It's already been tried as diagnostic method by few researchers. However, more studies are required to validate and optimize the assay for simple end-point detections.

3.6 | Rolling circle amplification

Another isothermal amplification-based assay, rolling circle amplification (RCA), is one of the oldest isothermal assays developed in 1995. The assay is based on the in vitro replication of circular DNA. In this method, the circular form of nucleic acid serves as the template for amplification. The amplification requires pair of primers, which complement the target in circular DNA as a template and polymerase enzymes like phi29 and *Bst* polymerase for the amplification of DNA or RNA. It can be performed at room temperature or higher.[52,53] Wang et al.[54] developed RCA for the detection of SARS virus and concluded that the method is a good alternative method for the detection of this virus. RCA has been developed for the detection of other viruses, including for the detection of Ebola virus.[55] This method has been drawing the attention of many investigators as it can be used as a diagnostic tool for detecting SARS-CoV-2.

4 | SENSITIVITY AND SPECIFICITY ISOTHERMAL AMPLIFICATION-BASED ASSAYS

The sensitivity and specificity of the assay is a crucial point for any detection method to become successful and qualify as POC diagnostic tool. The RT-qPCR is considered a gold standard for detecting SARS-CoV-2, as it offers a high degree of sensitivity and specificity. Therefore, an alternative method like isothermal amplification-based

assays must provide good sensitivity and specificity as compared to the gold standard. Among various isothermal amplification assays, more studies have been carried out using LAMP assay for SARS-CoV-2 detection. The assays are found to be highly sensitive, which is comparable with RT-qPCR assay. The study reports that isothermal amplification-based assay could detect one femtogram of the RNA, where RT-qPCR also demonstrated similar sensitivity.[14] Similarly, as low as 1–10 copies of RNA of SARS-CoV-2 virus can be detected by the isothermal assay.[56] Studies of Thi et al.[22] and Lu et al.[57] reported isothermal assays could detect 100 copies or less of RNA of SARS-CoV-2. However, the LOD can vary from one target gene to the other. In general, isothermal amplification-based assays were found to 80%–100% specific for the detection of SARS-CoV-2.[56] Similarly, the specificity of the isothermal amplification-based assays also been tested with the help of in silico analysis. For instance, Lamb et al.[14] computationally tested the specificity of LAMP assay by comparing it with other coronavirus sequences, showed 27%–54% mismatch with the primers used for novel coronavirus. Lu et al.[57] and Huang et al.[21] reported 100% specificity with no cross-reactivity with the other viruses. It is important to note that the specificity can change corresponding to the gene tested.

5 | RAPIDITY AND SIMPLICITY OF ISOTHERMAL AMPLIFICATION-BASED ASSAYS

The POC diagnosis should be rapid, simple and easy to handle. Various investigators have reported that isothermal amplification-based assays like LAMP assay are much more straightforward and relatively quicker than the gold standard RT-qPCR.[58] The isothermal amplification-based assays can be performed in less than an hour, and results can be interpreted much faster than RT-qPCR. In general, isothermal assays are reported to be an easy and less time-consuming method for the detection of viral pathogen.[59] The isothermal amplification-based assays can be further simplified by modifying the end-point detection process of nucleic acid amplicons. Various end-point detection methods have been optimized, such as using various dye indicators, labelled probe lateral flow, CRISPR-Cas etc, can be used as POC diagnostics to detect SARS-CoV-2.[39,40,43,58,60,61]

6 | LIMITATIONS OF ISOTHERMAL AMPLIFICATION-BASED ASSAYS

No diagnostic method can be perfect in all aspects as there can be some limitations. In general, all the isothermal amplification-based methods are considered to be highly sensitive. However, this high sensitivity can significantly increase the risk of contamination and, if not optimized well, often leading to false-positive results in samples and non-template controls.[50,62] Similarly, some isothermal assays were not found to be successful in detecting multiple targets in a single reaction.[33] On few occasions, primer mispairing and amplification inhibition are some of the disadvantages which will result in non-specific amplification of the products.[63] NASBA assay has its disability, which requires an initial denaturation step before the actual amplification. Also, this method sometimes fails when the temperature is more than 41°C due to enzyme degradation. Additionally, it can only amplify the target, which is more than 100 bp.[23,43] Some isothermal methods have restrictions over the type of templates used for amplification. For example, RCA method requires a circular DNA or RNA as a template.[33] Overall, if the isothermal amplification-based assays are optimised well, some of these limitations can be minimised and considered for the detection of SARS-CoV-2.

7 | CONCLUSION

COVID-19 testing based on the individuals showing symptoms of the disease is inadequate to identify asymptomatic carriers and develop containment strategy. Therefore, testing populations is important for diagnosis, epidemiology and surveillance. The isothermal amplification-based assays methods can be a good alternative for the sensitive detection of SARS-CoV-2. In a post-COVID pandemic situation, it will be important to monitor all patients coming into the hospital to differentiate SARS-CoV-2 positive patients to be kept in isolation wards. Therefore, various rapid isothermal amplification-based assays could be useful for this screening purpose. These methods can be delivered in a few hours, and the kits will find application for this purpose. Since the tests are based on isothermal amplification and visual detection, they can be performed in a simple lab set-up without the requirements of sophisticated instruments, which can be made available even at a rural primary health centre. This way, even people living in rural areas will have access to COVID-19 tests.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS CONTRIBUTION

Biswajit Maiti: conceptualized, writing-original draft, editing and finalization; Karanth Padyana Anupama: writing original draft and editing; Praveen Rai: writing-review and editing; Indrani Karunasagar: writing-review and editing; Iddya Karunasagar: writing-review and editing. All authors read and approved the publication of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the finding of this study are available from the corresponding author upon reasonable request.

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