



Isothermal nucleic acid amplification techniques: A comprehensive overview on their principle & applications as alternative to PCR

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ARTICLE INFO

Keywords:

Isothermal amplification
Diagnosis
LAMP
NASBA
HDA
RPA
RCA
Point-of-care testing

ABSTRACT

Isothermal nucleic acid amplification methods have gained prominence as a valuable innovation in molecular diagnostics. These methods function at a constant temperature without the requirement of thermal cycler apparatus, simplifying the amplification process and making them strong alternative to PCR, particularly in resource limited areas where quick, affordable, and straightforward nucleic acid amplification technique is needed. Over the past 20 years, various isothermal nucleic acid amplification techniques have been developed, among them loop-mediated isothermal amplification (LAMP), helicase-dependent isothermal DNA amplification (HDA), rolling circle amplification (RCA), strand displaced amplification (SDA), nucleic acid sequence-based amplification (NASBA) and recombinase polymerase amplification (RPA) are commonly used techniques to enable quick and easy detection of target nucleic acid. This approach simplifies molecular diagnostics, enabling broader access to genomic testing in various fields of research and clinical practice. Many researches and review articles have been published since isothermal amplification is developed. However, there is lack of comprehensive review to cover common isothermal amplification mechanisms, detection methods, and applications together. Therefore, this review provides a comprehensive overview on common isothermal amplification techniques as alternative to PCR by focusing on their advantages, reaction principles, product detection, applications in disease diagnosis, as well as IAM future perspective.

1. Introduction

Nucleic acids (DNA and RNA) are widely utilized biomarkers in scientific research, forensics, medicine, agriculture and other fields [1,2]. Currently methods for amplifying nucleic acid are classified into two: Thermocycling amplification and Isothermal amplification [3]. The first & widely used thermocycling nucleic acid amplification technique is polymerase chain reaction (PCR), invented by Kary B. Mullis in 1983 [4]. PCR use enzymatic reactions occurring in a thermocycler to create billion-fold duplicates of nucleic acids through cycling of three main phases: denaturation, annealing, and extension at various temperature. Denaturation is the initial phase set at 90-97 °C, followed by primer annealing at 50-60 °C and extension phase at 72 °C, where DNA Taq polymerase add nucleotides to the terminus of annealed primers to builds a new DNA strand in 5' to 3' direction that are complementary to the template strand, since

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previously generated DNA strands act as templates, the amount of DNA amplified by PCR grows exponentially, doubling at the end of each cycle [5]. Repetitive cycles eventually drop off after 30 to 40 cycles because of the reagent's limitation, pyrophosphate accumulation, PCR inhibitors found in the sample, extensive self-annealing and other factors [6]. The overall process is performed in a programmed thermal cycler apparatus that regulates the temperature and duration of every cycle. Despite PCR's wide range of application in biological and medical fields, it has certain limitation, including intensive sample preparation in order to eliminate amplification inhibitors, the need for skilled workers, difficulty in optimizing reaction condition & reliance on expensive thermal cycler equipment. As alternative to PCR isothermal amplification techniques (IAT) have been developed. IAT offers quicker target amplification & eliminate the need of costly materials [1,7–9].

In recent years, various isothermal amplification methods have been developed. Unlike PCR they operate at a constant temperature, eliminates the requirement of thermal cycler, has easy operation procedures, and requires less experienced personnel. The most frequently utilized isothermal amplification techniques includes strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA), recombinase polymerase amplification (RPA), helicase-dependent amplification (HDA), rolling circle amplification (RCA) and loop-mediated isothermal amplification (LAMP). These techniques differ in their specificity, sensitivity, and complexity (many enzymes or primers). They are advantageous than PCR in terms of speed, affordability, scalability, & portability [1, 10–13].

Nowadays, IATs are increasingly used in: pathogen diagnostic, food sector, identification of certain plant and animal species. & Also lately been used on environmental samples, such as wastewater, ambient water, animal and human feces, soil, plants, animal tissues and for genetic testing [14–23].

2. Isothermal nucleic acid amplification methods

2.1. Loop mediated isothermal amplification (LAMP)

LAMP was developed by Notomi et al., in 2000. It amplifies DNA under isothermal condition with great efficiency, specificity, and speed. The amplification is carried out by incubating the mixture of samples, six primers precisely built to recognize eight different locations on the target gene, dNTPS, $MgSO_4$, LAMP buffer and Bst polymerase, which has strand displacement activity under isothermal temperature that ranges between 60 °C up to 65 °C [24].

There are two phases in LAMP: non-cyclical and cyclical phase. The non-cyclic stage produces a DNA with stem-loops at both ends, which used as the template structure for cyclical phase. The double strand DNA become in a state of dynamic equilibrium approximately at 65 °C allowing forward Inner Primer (FIP) to anneal to a complementary sequence at the target DNA to initiate the non-cyclical phase, extension from the site of annealing proceeds by Bst polymerase, results the displacement of single stranded DNA & synthesis of complementary strand. The newly generated DNA strand is then displaced by the forward outer primer (F3) by annealing to the F3c area exterior to the FIP location. This results the production of single stranded DNA with a dumbbell-shaped at 5' terminus, which used as a template for the backward Inner Primer (BIP) to be anneal at a complementary B2c region & elongated by the Bst polymerase, followed by annealing of the backward outer primer (B3) to the B3c area outside of the BIP site to start displacement of previously synthesized strand by bst polymerase, this leads to the formation of a dumb bell-shaped DNA at the 3' termini [24,25].

At the completion of the non-cycling phase DNA strand with stem loops at each terminus is generated and used as a template for cyclical phase. During the cyclical phase, internal primers are annealed to the stem loop DNA & starts DNA synthesis with strand displacement. In addition, loop primers: forward loop primer (FL) and backward loop primer (BL) can be used to accelerate the amplification activity, they binding to additional sites that internal primers cannot access. The entire process generates up to $\sim 10^{12}$ copies of DNA within an hour. As compared to PCR, LAMP detects & amplifies fewer copies of the target DNA more efficiently under isothermal conditions. It is robust alternative method for PCR due to: quick amplification, ease of use, easy detection, affordability, sensitivity, & specificity. Nowadays, LAMP is increasingly used in a variety of sectors including the medical, agricultural, and food [8, 24–32]. Even though LAMP method is quite useful, there are some restrictions that may make it difficult to use including the complexity of the LAMP primer design [27,33]. Furthermore, LAMP's final product is less versatile than PCR, making it unsuitable for cloning [34,35].

2.1.1. LAMP primer

Primer design is among the most important aspects influencing the effectiveness and quality of the nucleic acid amplification techniques. The following primer sets are employed in LAMP; external forward(F3) and backward primer (B3) and forward internal primer (FIP), backward internal primer (BIP); The internal primers are complementary to two distant places on the template (on the sense strand and the antisense strand) and have a length of (40–49 bp), to allow the external primers binding with the target more gradually than the internal primers, they are applied to the reaction mix at reduced concentrations and they have shorter length (20–24 bp), in addition optional loop primers, loop primer forward (FL) and loop primer backward (BL) are also used, they are made to bind to extra sites that internal primers are unable to access, so that they accelerate the amplification activity. The six kinds of primers are designed depending on the eight different locations of target gene, B1, B2, B3, and BLP found at the 5' area and the F3c, F2c, F1c and the FLP sections at the 3' side [36,37].

FIP contain F2 region, which is complementary to F2c area at the 3' end, and the F1c region, which is in the same sequence at the 5' end. Forward outer primer(F3) is complementary to the F3c area, BIP is composed of the B2 region, which is complementary to the B2c region, located at the 3' end, and the same sequence of B1c region at the 5' end. Backward outer primer (B3) is complementary to the B3C area, the FLP and BLP primer sequences are complementary to the sequences separating the F1&F2 and B1&B2 sections,

respectively [24].

2.1.2. Detection of lamp products

LAMP has capacity to synthesize enormous quantity of DNA, which are a combination of stem-loop DNAs with several inverted target repeats and looped formation that have a cauliflower-like appearance. There are two methods that can be employed to detect the LAMP product. The first is based on evaluation of the result while the amplification procedure is ongoing (real-time) & the second is based on evaluation of results after amplification reaction (end-point methods) [38,39]. The majority of visualization techniques are based on end point methods. The common end point detection method is agarose gel electrophoresis, allows visualization of LAMP product as a ladder like structure. Turbidity is another alternative method for detecting LAMP amplification. During the LAMP reaction, magnesium pyrophosphate ($Mg_2P_2O_7$) is produced as a by-product, resulting the formation of a white precipitate. This precipitate is visible in positive reaction tubes, which can be observed with the naked eye, whereas negative tubes remain clear. Also, addition of nucleic acid binding fluorescent dyes like SYBR Green or Calcein in the reaction mixture allows naked-eye detection of the product through color change. Furthermore, real time detection methods, including real-time turbidimeter & real-time fluorescence has been used to evaluate the result during the reaction is ongoing [40–44].

2.2. Helicase dependent amplification (HDA)

HDA is one of the simplest isothermal amplification techniques that mimics an in vivo DNA replication process. A helicase enzyme is used in place of heat, as in PCR, to isothermally unwind double strand DNA. This approach was created in 2004 by Vincent and his colleagues. The earliest studies used thoughtfully researched helicase II UvrD from *E. coli*, which unwinds dsDNA in 3' to 5' direction by breaking the hydrogen bonds between complementary bases by using energy derived from adenosine triphosphate hydrolysis [45–47]. Helicase II UvrD is a protein that assists repairing of DNA mismatches in *E. coli* cells by collaborating with the methyl-directed mismatch repair (MutL) protein, which activates helicase action. Initially developed HDA needed two accessory proteins in addition to helicase enzyme to facilitate unwinding of the dsDNA. The first accessory proteins is a single-stranded DNA-binding protein (SSB) used to stabilize the separated ssDNA and stop the complimentary strands from re-assembling and the second one is MutL protein used for enhancing helicase activity [46].

Tat the initial stage of the reaction the dsDNA become isothermally unwound by the action of helicase with MutL protein and stabilized by SSB protein, then forward and backward primers anneal to 5' and 3' end of the target sequence, followed by extension of annealed primers by DNA polymerase to synthesizes complementary strand [48]. This process is repeated in successive cycles, leading to the exponential accumulation of the amplification product within 60-90 min. The first study on HDA used an *E. coli* UvrD helicase/DNA polymerase I Klenow fragment pair to amplify DNA exponentially at 37 °C, resulting in a mesophilic version of HDA. The mode of action of UvrD helicase require substantial molar excess relative to the substrate to unwind dsDNA. However, its limited speed and processivity significantly reduce the efficiency of HDA, particularly when amplifying larger target. Eventually, the thermophilic variant of HDA (tHDA) was developed, which enabled for the amplification to be done at a temperature range of 60–65 °C. This was made possible by the helicase Tte-UvrD which is thermostable derived from *thermoanaerobacter tengcongensis* in conjunction with Bst-DNA polymerase. The components of the tHDA system are simple as contrast to mHDA, as it does not need MutL protein. Furthermore, the tHDA exhibits enhanced efficiency, sensitivity, and specificity [45,47,49–51].

HDA was further improved by linking the UvrD helicase and Bst-DNA polymerase to create helimerase, novel bifunctional protein that speeds up DNA synthesis. In comparison to the two separate proteins, this complex showed improved processivity and enabled the amplification of targets that were substantially long up to 2.3 kb. Nevertheless, this novel enzyme is not yet commercially accessible, and additional research needs to be done to streamline and lower the cost of production [46,47,51]. HDA amplifies nucleic acids exponentially using two flanking primers. Several primer combinations are frequently designed by utilizing online programs in order to get high yield of a target-specific amplicon. HDA can also be integrated with reverse transcription for the amplification of RNA. HDA has great promise as a substitute for PCR and developing devices that enables point-of-care examination based on HDA amplification principle will be beneficial in environments with limited resources. Due to the fact that HDA has a fairly straightforward reaction configuration and a PCR-like reaction scheme that makes it possible to adapt existing PCR method to the HDA framework is one of its main benefits over other alternative isothermal amplification approaches [52,53].

2.2.1. Detection of helicase dependent amplification

End point gel electrophoresis and fluorescence based real time techniques can be used detect HDA amplicons. The initial version of real-time HDA was created with EvaGreen, not-cytotoxic fluorescent and non-mutagenic intercalator dye that only glows when attached to dsDNA, ensuring minimal background signals, it also exhibits a lower level of amplification inhibition than SYBR Green, even if there is commercially accessible real-time equipment for HDA, Portable detection devices are still being developed [53–55].

2.3. Nucleic acid sequence-based amplification (NASBA)

NASBA is a sensitive transcription-depending isothermal amplification technique initially developed in 1991 for the identification of RNA targets. This method doesn't require specialized equipment to operate and accomplish up to one million RNA copies in 90 min with just four to five cycles [56–58]. NASBA is mediated by three enzymes: T7 DNA-dependent RNA polymerase (DdRp), RNase H, reverse transcriptase (RT) of the avian myeloblastosis virus (AMV) and two primers (P1 & P2). The forward antisense primer (P1) has a non-complementary promoter sequence at the 5' end for the corresponding T7 RNA polymerase & the second primer (P2) is a reverse

primer intended to hybridize with cDNA produced by P1 [59].

At the initial phase of the reaction process P1 primer hybridizes with the target RNA at 65⁰c and elongated by AVM-RT to produces a complementary RNA/cDNA hybrid. Subsequently, breakdown of RNA from hybrid RNA/cDNA by RNase H takes place, forming a single-stranded cDNA, which allows the binding of P2 primer at 41⁰c and forms subsequent dsDNA. RNA polymerase, which is limited to binding on double-stranded promoter region, recognizes the T7 promoter and transcribe the relevant RNA strands resulting in the production of single strand antisense RNA copies, each transcribed RNA strand annealed by the P2, & an RNA-/cDNA hybrid synthesized using AMV reverse transcriptase. P1 can attach to the cDNA after the RNA-strand of the resultant hybrid is broken down by RNase H, and AMV reverse transcriptase then extends p1 & forms double strand cDNA. Due to the presence of the T7 RNA promoter in the resulting double-stranded DNA, transcription produces copies of the RNA. All newly produced RNA-can be utilized as a template to generate more cDNA intermediates, which in turn create more -RNA [57,59].

In addition to RNA, DNA can also be amplified using NASBA; however, the process for DNA amplification requires two denaturation steps. Initially, the double-stranded DNA is denatured at 95 °C, followed by the binding of P1 to ssDNA & subsequent extension by AMV reverse transcriptase to generate dsDNA. Subsequently, P2 can bind to P1's extension product following the second denaturing procedure. The second denaturation process rendered all three enzymes inactive, therefore they must be introduced again [56,60]. NASBA has certain advantages including in diagnosing RNA viruses & requirement of minimal power source due to the modest reaction temperature of 41 °C required for the process. However, it has certain limitation such as the need of denaturation procedure in case of DNA amplification make these techniques not completely isothermal and reaction enzymes need to be added individually due to their thermolability. Furthermore, the range of nucleotide base pairs that can be amplified efficiently is 120-250 [61,62].

2.3.1. Detection of NASBA product

The primary method of NASBA amplicon detection is end-point based. The product can be conventionally isolated using gel electrophoresis, NASBA is often used in conjunction with a lateral flow device for RNA virus such as HIV detection in order to achieve faster end-point detection. Furthermore, product can be detected using real-time techniques such molecular beacon probes or SYBR green dyes [58,63].

2.4. Rolling circle amplification (RCA)

RCA was initially developed in 1995. It amplifies the target DNA by means of a polymerase enzyme possessing strand displacement activity. In the field of clinical and biomedical research, this method has shown as a robust alternative to PCR. Simplesness, high sensitivity, and high specificity are just a few benefits of using the RCA. The main components of RCA are a single-stranded circular template (CT), dNTPS, a minimum of one primer and a polymerase enzyme with strand displacement activity. The amplification process creates many amplicons of circular nucleic acids, such as plasmids and the genomes of bacteriophages [64–66]. A single primer anneals to the circular DNA template to start the amplification. Afterwards, commonly used Phi29 bacteriophage DNA polymerase (ϕ 29DNAP) gradually expands throughout the template, the amplification of DNA occurs as the strand created by the strand displacement activity of ϕ 29DNAP is constantly replaced by the newly created strand, the amplification continues until some reactants runs out [57]. Long Tandem repeats of single-stranded target DNA sequence are formed during RCA, It can be broken down into each unique short oligonucleotide by using enzymes [66,67]. RCA can amplify single copy of the target DNA in 100,000 non-target DNA duplicates [68].

One of the other RCA versions are ligation-RCA (L-RCA), It relies on padlock probe which allows amplification of linear single-stranded template DNA through RCA reaction. In L-RCA the padlock probe binds with the target and ligated by DNA ligase at each terminal to create circular DNA template. The RCA primer anneal to created circular template then the DNA polymerization process starts by the DNA polymerase, the amplification product consists of long single-stranded DNA concatemers, with repeated sequences that are complementary to padlock probes. The RCA is carried out at low temperatures, roughly at 23 -60 °C for around 60 min [69,70]. A significant advancement in RCA technology was the transformation of the initial linear rolling circle amplification method into an exponential. To achieve this, at least one more primer that is complementary to the previously synthesized product is used. Hyper-branched RCA is the most prevalent kind of exponential RCA. These variants uses forward and reverse primers to amplify DNA exponentially, normal RCA products are long ssDNAs, but HRCAs products are double-stranded DNA (dsDNA) [71,72]. RCA offers a lot of clear benefits. Firstly, the ligation of a padlock probe requires precise complementarity, which confers high specificity to the RCA reaction. Secondly, multiple primer introduction makes it simple to create exponential amplification, which results in high sensitivity. Thirdly, functional nucleic acids including aptamers, DNazymes, and restriction enzyme sites can be produced by modifying circular templates in RCA products [73]. Nowadays, RCA has been employed more frequently in the detection of DNA/RNA due to its ease of use, robustness, specificity, and high sensitivity [74].

2.4.1. RCA product detection

Numerous technologies are available for monitoring and detecting of RCA products. The method for RCA product analysis is gel electrophoresis. In addition, fluorescence-based approach such as complementary strand induced fluorescence response by binding of RCA product with fluorescence dye and lateral flow assay are among the method of detection [74–76].

2.5. Strand displacement amplification (SDA)

Strand displacement amplification (SDA) was initially described in 1992 by Walker and his colleagues. It has the ability to amplify a

target sequence up to more than 10^9 copies within 2 h at 37 °C [77]. SDA has been used in various fields, such as in the identification of infections agents, genetic disorders and cancer [78–81]. The SDA process is based on the continuous nicking, extension, and displacement, facilitated by restriction endonuclease *HincII*, exonuclease-deficient DNA polymerase, bumper primers (B1 and B2) and SDA primers (S1 and S2). In SDA primers; linker, nicking endonuclease recognition site & complementary sequence to the target are present. Bumper primers are complementary to the target that are found at upstream of the SDA primers target [82].

The amplification starts by denaturation of dsDNA at 95 °C, allowing the S1 and S2 primer to anneal on each ssDNA, subsequently extension of the primers proceed. Each of the newly formed strands from s1 and s2 extension are displaced by bumper primers B1 and B2 respectively, resulting in double-stranded DNA and displaced single strands from S1 and S2 extensions. Subsequently, B2 and S2 anneal to S1 extension and B1 and S1 anneal to S2 extension, followed by extension and displacement steps as in the previous step, The extension in both strands is done simultaneously by exo-klenow DNA polymerase in the presence of dGTP, dCTP, TTP and thiol-modified dATP (dATP α S). As a result, two fragments with the hemiphosphorothioate *HincII* site on both ends and another pair with it only on one end were formed. Due to modified dATP incorporated into the newly produced DNA strand, endonuclease nicking of the strand at any site is prevented. This round of annealing and extension continues until a dsDNA with both strands bearing *HincII* restriction sites. The strands are then nicked by the endonuclease enzyme the endonuclease nicks the unmodified original strand extended from the primers, followed by polymerase extension of the strand at the nick, leading to multiple circles of nicking, extension and displacement. The displaced strands then form the template for subsequent amplification which culminate to more rapid target amplification [82–84].

SDA's incapability to effectively amplify lengthy target sequences is its primary disadvantages [85]. Furthermore, modified version of Strand Displacement Amplification (SDA) was developed using nicking endonucleases, which are engineered versions of restriction enzymes such as *Nt.BsmAI*, *Nb.BsmI*, *Nb.BsrDI*, *Nt.BspQI*, *Nb.BtsI*, *Nt.AlwI*, *Nt.BbvCI*, and *Nt.BstNBI* to improve the accuracy of site-specific cleavage of double-stranded DNA sequences. Unlike traditional methods, this approach does not require modified nucleotides like dATP α S at the cleavage site. These enzymes introduce nicks in the DNA, allowing Klenow fragment or Bst polymerase to initiate strand replication and displace the downstream strand. The improved SDA method results in rapid and exponential DNA amplification, achieving up to a 10^9 -fold in less than 30 min at temperatures ranging from 37 °C to 42 °C [82,86–88]. Although, SDA was previously linked to DNA amplification, it has been modified to detect RNA such as, microRNAs(miRNAs), which used as a target for the diagnosis of disease. The miRNA target may initiate two cycles of nicking, polymerization, and displacement processes with the Klenow fragment, the nicking enzyme *Nt.AlwI*, and two primers. These reaction cycles exponentially increased the target miRNA and produced detectable dsDNAs [89].

2.5.1. SDA product detection

Detection of the SDA product involves real time and endpoint methods, gel electrophoresis, fluorescence methods are among commonly used method, the SYBR Green utilized in detection of amplification product, however high SYBR Green concentrations can prevent SDA amplification. Also, molecular beacons, lateral flow assay, can be used for the detection of SDA product [63,80,90].

2.6. Recombinase polymerase amplification (RPA)

Recombinase polymerase amplification (RPA) is invented by Piepenburg et al., in 2006 [91]. It was anticipated that it would replace PCR because of its benefits including easy operation, quick amplification speed & no need of thermal cycler. It takes about 20 min to amplify 1–10 target copies of DNA under a temperature range of 37 to 42 °C and it requires little sample preparation, single-stranded DNA, double-stranded DNA, miRNA and cDNA can all be amplified by using RPA [92,93].

RPA uses a recombinase enzyme which facilitates the binding of the primer to the complementary region, DNA-binding proteins (SSB) and DNA polymerase. In the initial report of RPA, recombinase protein uvsX from T4-like bacteriophages, *Bacillus subtilis* PolI DNA polymerase & SSB are used. The amplification process is initiated by the binding of recombinase protein uvsX from T4-like bacteriophages to the primers with the existence of polyethylene glycol & ATP to create a recombinase-primer complex. Subsequently, the complex proceeds with searching of complimentary sequence on double-stranded DNA and induces strand invasion at the corresponding complimentary region by the primer. Single-Strand Binding (SSB) proteins subsequently attach to the displaced DNA strand to stabilize the displaced strand and ensure that the inserted primer is not being ejected by branch migration. The recombinase then disassembles, allowing a strand-displacing DNA polymerase to attach to the primer's 3' end and extend it in the presence of dNTPs. Exponential amplification is achieved by repeating this procedure cyclically [91,92]. Although it was formerly thought that RPA required specially created primers with a length of 30-35 bases, multiple reports have shown that regular PCR primers can be used and effective amplification can be accomplished [94,95]. RPA can also amplify RNA by adding reverse transcriptase to the same reaction tube [96].

2.6.1. Detection of RPA product

Several detection techniques, including real-time fluorescence detection and end-point lateral flow strips, have been effectively combined with RPA [92]. As a Realtime detection, fluorescent probes and a fluorimeter making it easier to detect & quantify the amplicon. Also generalized intercalating fluorophores like Eva Green or SYBR Green are able to be used for real-time detection [97,98].

3. Isothermal nucleic acid amplification integration with portable devices

Isothermal nucleic acid amplification methods are now being combined with a compact portable device, such as microfluidic chips,

capillary platforms, and test strips. There are commercially available devices and diagnostic kits that are designed for point-of-care (POC) testing [10]. Isothermal microsystems, which have advantages over PCR can be designed to be simple, portable, and low energy-demanding without requiring thermal cycling. This allows for process automation and integration within a single device. Microfluidic technology is a compact portable device which has a significant advantage, includes low sample, reagent usage, high portability and quick assay times. It reduces the risk of sample contamination and improves detection capability [99].

Currently, microfluidic platforms are integrated to HDA, RPA, NABSA, LAMP, SDA & RCA for identifying the target nucleic acid [100–103]. Capillary tubes are the other simple portable devices, unlike microfluidic chips, which involves a complex and costly microfabrication, capillary tubes can be prepared easily and affordably. They have been used to conduct isothermal amplification reactions in small volumes. Liu et al. were the first to introduce an integrated capillary-array microsystem designed for the extraction, amplification, and detection of DNA from *Mycobacterium tuberculosis* [104].

Test strip paper is the other simple device technique which gained growing interest due to its portability, availability, easy fabrication, and low cost particularly to be used for point-of-care diagnostic RCA, RPA, SDA and NASBA are integrated with test strip paper-based assay method. [105–107]. One of the other common methods for simple product detection is a lateral flow strips which allows quick detection of the target. This technique commonly used with LAMP, HDA, RPA, and RCA, involves binding of antibodies to a test strip of paper that complex with probes that are unique to particular nucleic acid sequences. The amplicons will be bound to the antibody probe while an isothermal amplification output is applied, the antibody probe linked to a biotin ligand on the testing line on the strip generates a visible signal which indicates a qualitative result [17,108–110].

4. Applications isothermal amplification in disease diagnosis

Infectious diseases are the primary cause of both morbidity and mortality globally. The first step towards treatment, as well as disease control and prevention is an accurate and immediate diagnosis. Reliable diagnostic methods are essential for identifying diseases, providing appropriate care, and managing population outbreaks. Choosing the appropriate diagnostic techniques for quick pathogen identification is crucial for both public health programs and clinical diagnosis. Conventional diagnostic methods are not suitable for effective diagnosis because they usually have poorer detection rates, longer analysis periods, and less automated. The ability to detect infectious disease pathogens early and efficiently has been greatly improved by recent developments in molecular detection technologies, which offer improved sensitivity, specificity, quicker detection, and more automation. Nucleic acid amplification methods are used to diagnose disease by detecting DNA or RNA [111–113].

Nucleic acid testing is extremely necessary to enhance the identification of causative agent. The current generation of molecular tests, which are primarily PCR-based, is not very useful in point-of-need or resource-constrained environments. Therefore, developing robust, portable, quick, sensitive, and reasonably priced systems for the early identification of the target is highly desirable in order to treat and control infectious diseases [23,53,114].

The implementation of nucleic acid amplification methodologies has led to a greater sensitivity, accurate, and quick diagnosis of diseases. Although commonly used, NAAT like polymerase chain reaction (PCR) which rarely accessible in areas with low resources, nucleic acid can be detected in samples quickly, sensitively, specifically, easily, and affordably via isothermal amplification (IA) techniques [115]. Furthermore, nucleic acid can be effectively detected from a variety of sources, including hair follicles, tissue biopsies, buccal swabs, urine samples, and dried blood spots. Specimens from buccal swabs and hair follicles can be collected non-invasively and are appropriate for regular genetic analysis. Biopsy samples provide high-quality genomic DNA for confirmatory testing, while urine and dried blood spot samples are valuable for minimally invasive diagnostics and large-scale screening programs. Isothermal amplification's suitability for several specimen types shows its potential for forensic analysis, point-of-care testing, and population level genetic surveillance, especially in environments with low resources where traditional PCR equipment would not be easily accessible [116]. The World Health Organization (WHO) has indicated the requirements for point-of-care (POC) testing into their assured suggestions, which are intended to be, affordable, specific, fast, reliable, highly sensitive, as well as simple to perform. Therefore, it has been established that isothermal amplification techniques are suitable for POC applications and meet the WHO's assured requirements [7,117].

4.1. Loop-mediated isothermal amplification (LAMP) in disease diagnosis

LAMP is a technique that have ability in quick, accurate, and affordable diagnosis of infectious diseases. Several investigations that have been conducted with LAMP successfully show off its impressive contribution in addressing the gap in molecular diagnostics. LAMP has been widely applied for the rapid detection of infectious diseases including identification of viruses, bacteria, and parasites pathogens [28]. In developing nations, malaria poses a serious risk to the health of pregnant mothers as well as children. Since the signs and symptoms of malaria coincide with numerous other diseases, it's critical for patients to receive an effective diagnosis as soon as possible. Since PCR can detect & distinguish different kinds of malaria. Nevertheless, it is not feasible for peripheral laboratories in developing nations due to the time, cost, and infrastructure requirements [118,119].

The LAMP method is capable of identifying *P. falciparum* from blood with a sensitivity of about 6 parasites/μl blood, nearly ten times greater than traditional blood smear test which requires about 50 parasites/μl blood, in low-resource healthcare facilities in developing nations, the LAMP method with straightforward heating of blood specimens is a possibility used for identification of *P. falciparum* [120]. Additionally, LAMP primer pairs which allow genus-level diagnosis and the differentiation of all four species have been produced by Han et al. [121]. Generally this assay may offer a substitute for traditional microscopy in rural health center in developing nations enabling the routine detection of malaria [122].

Another, most common infectious disease is tuberculosis (TB). Its incidence has been rising in developing nations. Lack of accessible, low-cost diagnostic techniques with greater sensitivity than the sputum smear test is one of the reasons why tuberculosis control has failed in developing nations. Despite the fact that NAAT techniques to combat tuberculosis have become widespread in the developed countries, this method is not sufficiently straightforward and reasonably priced to be used in regional laboratories in developing nations. A LAMP assay that able to identify mycobacterium tuberculosis, *M. avium*, and intracellular has been developed by Iwamoto and colleagues [123–125]. Furthermore, significant pathogens such as human papilloma virus, COVID-19, Cryptosporidium oocysts, Legionella mumps virus, measles virus can now be diagnosed using LAMP testing [126–132] (Table 1). Shows additional examples of disease detected by LAMP.

4.2. Rolling circle amplification application in disease diagnosis

Several researches that have been conducted on RCA shows the identification of single nucleotide polymorphisms (SNPs), microRNAs (miRNA), bacterial and viral nucleic acids can all accomplished with RCA with high sensitivity and specificity [143–145]. As example, Hamidi and his colleagues designed an RCA technology in 2015 which integrates hyperbranched RCA (HRCA) with fluorometric detection to identify H5N1 influenza RNA virus with a great sensitivity [146]. Also, wang & his colleagues established the RCA detection techniques for the severe acute respiratory syndrome coronavirus (SARS-CoV). They designed an HRCA-based method for detecting the single-stranded RNA coronaviruses [145] (Table 2). Shows additional examples of RCA application in disease detection.

4.3. Recombinase polymerase amplification in disease diagnosis

RPA has been widely used for the identification of pathogen including viruses, bacteria, parasites and fungus, drug resistance gene detection and for genetic testing from the clinical sample quickly & accurately. RPA appears to be more common in molecular diagnosis. The use of this method in nucleic acid detection was especially even more encouraged in 2019 SARS-CoV-2 outbreak [155–157]. As example, the Plasmodium falciparum 18SrRNA gene fragment was amplified by using RPA-LF. The findings of the detection were acquired at 38 °C in less than 20 min. It advances worldwide malaria control efforts by detecting Plasmodium falciparum genomic DNA as few as 100 fg, making it ideal for on the spot detection in remote locations [158]. *Haemophilus influenzae* with and without capsules can be distinguished using the RPA-lateral flow strips (RPA-LFS) method, which was created by Wang et al. and has a detection limit of 1 cfu/μL. Additionally, *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* are detected by RPA-LFS [159–161]. Recently, Helite and his colleagues developed the RPA-LFS for rapid detection of SARS-CoV-2, having a detection limit of 35.4 viral cDNA nucleocapsid gene copies/μL. Reverse transcription-qPCR reference test and RPA-LFS showed 100% consistency [162] (Table 3). Shows additional examples of RPA application in disease diagnosis.

4.4. Helicase dependent amplification in disease diagnosis

Helicase-dependent amplification (HDA) has been effectively used since its introduction in 2004, it allows molecular-based detection of pathogens quickly and accurately. The majority of HDA's applications are focus on clinical setting, where quick and affordable tools are becoming more and more necessary to lower the rising expense of diagnosis [53,171]. Table 4 Shows examples of HDA application in disease diagnosis.

4.5. Nucleic acid sequence-based amplification (NASBA) in disease diagnosis

NASBA is used in molecular diagnostics to detect RNA/DNA sequences with high sensitivity and specificity. [179]. This technique is highly valuable pathogen detection. Examples of NASBA applications in disease diagnosis are provided in (Table 5).

Table 1

= Shows Example on Applications of Loop Mediated Isothermal Amplification for disease detection.

Target	Sample	Template	Detection method	Ref
Middle East Respiratory Syndrome-Coronavirus (MERS-CoV)	Nasopharyngeal aspirate sample	RNA	Fluorescent dyes	[133]
Mycobacterium spp	Sputum & cultured bacteria	DNA	Fluorescent dyes	[134]
<i>Vibrio cholerae</i>	feces	RNA	Fluorescent dyes	[135]
Adenovirus	Tissue samples	DNA	Real time turbidity	[136]
Entamoeba histolotica	Spiked feces	DNA	Turbidity, Fluorescent dyes	[137]
Visceral leishmaniasis	Blood	DNA	Turbidity Fluorescent dyes Gel electrophoresis	[138]
<i>E. coli</i> 0157	Cultured bacterial sample	DNA	Gel electrophoresis	[139]
shigella	Stool	DNA	Gel electrophoresis, Turbidity	[132]
<i>Mycobacterium tuberculosis</i>	Sputum	DNA	Gel electrophoresis	[140]
Methicillin resistant staphylococcus aureus (MRSA)	Blood Cultured sample	DNA	Gel electrophoresis	[141]
β-thalassemia mutation (HBB gene)	Whole Blood	DNA	Fluorescent based	[142]

Table 2

Shows an example of rolling circle amplification applications in disease diagnosis.

Target	Sample	Template	Detection method	Ref
Ebola Virus	Blood	RNA	padlock probes	[147]
miRNA/cancer biomarker	Serum	RNA	Fluorescent labeled probes	[148]
<i>Mycobacterium tuberculosis</i>	Sputum	DNA	Fluorescent labeled probes	[149]
Shigella	Stool	DNA	Gel Electrophoresis	[150]
<i>E. coli</i> 0157	Food	DNA	Fluorescent dyes	[151]
Tumor-associated microRNA	Serum, plasma	RNA	Fluorescent based detection	[66]
Covid 19	Sputum	RNA	fluorescent labeled probes	[152]
Listeria monocytogens	From Culture	DNA	Fluorescent based detection	[153]
Malaria	Blood	DNA	Microfluidic device	[154]

Table 3

Shows additional examples of RPA application in disease diagnosis.

Target	Sample	Template	Detection	Ref.
<i>Cryptococcus neoformans</i>	CSF	DNA	Lateral flow assay	[14]
<i>Schistosoma haematobium</i>	Urine	DNA	Real time fluorescence	[163]
multidrug-resistant tuberculosis	Sputum	DNA	Fluorescence dye	[164]
hepatitis B virus	Blood	DNA	Lateral flow assay	[165]
Respiratory syncytial virus	Sputum	RNA	Lateral flow assay	[18]
<i>Rickettsia typhi</i>	plasma	DNA	Real time fluorescence	[166]
<i>Brucella</i>	Serum		Real time fluorescence	[167]
<i>Listeria monocytogenes</i>	Blood	DNA	Fluorescence dye	[168]
<i>Mycobacterium tuberculosis</i>	Sputum	DNA	Fluorescence dye	[169]
Single nucleotide variant screening (SNV)		DNA	Real time fluorescence	[170]

Table 4

Shows example on applications of helicase dependent amplification for disease diagnosis.

Target	Sample	Template	Detection Method	Ref
<i>E. coli</i>	Culture	DNA	Gel electrophoresis	[172]
<i>B. malayi</i>	Blood	DNA	Gel electrophoresis	[47]
<i>S. aureus</i>	blood culture	DNA	LFD	[173]
<i>C. difficile</i>	Stool	DNA	LFD	[174]
Salmonella	Culture	DNA	LFD	[175]
<i>P. falciparum</i> and <i>P. vivax</i>	Blood	DNA	LFD	[176]
<i>Mycobacterium tuberculosis</i>	Sputum	DNA	LFD	[177]
<i>T. vaginalis</i>	vaginal swab	DNA	Fluorescence probe	[55]
HPV-16	Cervical swab	DNA	Realtime Fluorescence	[54]
Covid 19	Nasal Swab	RNA	LFD	[178]

4.6. Strand displacement amplification (SDA) in disease diagnosis

Strand Displacement Amplification (SDA) is effective for detecting infectious pathogens due to its rapid and sensitive isothermal amplification of target DNA in clinical samples. The method's ability to detect pathogens directly from biological samples without extensive sample preparation makes it ideal for clinical diagnostics. Several research have assessed the effectiveness of strand displacement amplification (SDA) in detection of pathogen such as *C. trachomatis* using samples: urine, endocervical, and urethral swabs [187,188]. Furthermore, staphylococcus aureus gene, NO-inducible l-lactate dehydrogenase (ldh1), has been detected

Table 5

Shows additional examples of NASBA in pathogen detection.

Target	Sample	Template	Detection Method	ref
Epstein-Barr virus (EBV)	biopsies	RNA	gel electrophoresis	[180,181]
<i>H. influenzae</i>	whole blood	DNA	molecular beacon probe	[182]
<i>N. meningitidis</i>	whole blood	DNA	molecular beacon probe	[182]
<i>S. pneumoniae</i>	whole blood	DNA	molecular beacon probe	[182]
Rhinovirus	Cell cultured	RNA	biotin-labeled oligonucleotide probes	[183]
<i>Mycobacterium leprae</i>	Skin biopsies	DNA	Fluorescence based	[184]
Leishmania parasite	Skin biopsies	DNA	Molecular probes	[185]
Human papilloma virus	Cervical sample	RNA	Real-time Fluorescence	[179]
Covid 19	Nasal Swab	RNA	Molecular Beacon	[186]

successfully using iSDA coupled with lateral flow [82] (Table 6). shows some additional examples of SDA in disease diagnosis.

5. Quality assurance in isothermal nucleic acid amplification

Quality assurance in isothermal nucleic acid amplification assays requires comprehensive control of pre-analytical and analytical variables to ensure clinical reliability. Assessment of nucleic acid concentration, purity, and potential inhibitors is essential for the high performance of the assay. Analytical quality control should be performed by including positive, negative, and internal amplification controls to detect contamination and reaction failure. Assay validation must establish limit of detection, analytical specificity, precision, reproducibility, and overall diagnostic performance against reference methods. Clinical implementation of isothermal amplification is further supported by temperature calibration, reagent commercialization, establishment of standard operating procedures, and involvement in external quality evaluation programs [192–194].

6. Comparative summary of isothermal amplification and PCR

Isothermal amplification methods are generally considered cost-effective because they require minimal instrumentation. These methods run at a constant temperature, which eliminates the need for expensive thermal cyclers and maintenance expenses compared to conventional PCR. The majority of isothermal amplification method performed with portable platforms or simple laboratory heating equipment, which makes them cost-effective for resource-limited environments. In general, their infrastructure requirements, easy detection method, and simplicity contribute to lower operating costs as compared to conventional molecular diagnostic techniques. The Summarized comparison of PCR and isothermal amplification are summarized in (Table 7).

7. Future perspective on isothermal amplification

Isothermal amplification techniques to detect pathogens in water, food, environment, clinical specimens and for genetic testing are now being implemented and commercialized. However, reaching technological maturity and wider reagent and enzyme commercialization are still prerequisites for the success of isothermal amplification techniques. Currently, being developed and commercialized isothermal amplification techniques have a benefit including rapid detection, simplicity of usage, and less need of advanced laboratory infrastructure. However, in order for isothermal amplification techniques to become widely used and ensure technological maturity a number of obstacles still need to be overcome. Key prerequisites include the optimization of reagent and enzyme production, ensuring consistent performance across diverse applications, and the establishment of standardized protocols. Researchers are actively working on improving the precision and reliability of these methods through better enzyme formulations, optimized reaction conditions and to address current limitations for enhancing the performance of the methods. The nearest future should witness more compact hand-held diagnostic technologies that operate isothermal principles and still combine such simplicity with accuracy and rapidity.

8. Conclusion

The development of innovative isothermal nucleic acid amplification techniques has a significant importance across various fields. The ability to amplify nucleic acids at a constant temperature without the use of a thermocycler makes isothermal amplification well suited choice for point-of-care (POC) testing in public health & biological research. Although they are not widely commercially accessible, the majority of alternative technologies have the potential to be use as alternative to PCR, but they still need to undergo rigorous validation test. Given the shared benefits and drawbacks of isothermal nucleic acid amplification techniques, proposing a single technique as a universal pathogen detection platform remains challenging. For point-of-need applications, the LAMP method is currently more prevalent technique among other isothermal amplification method. Generally, IATs provides affordable option for molecular diagnostics, allowing quick and precise identification of infectious and genetic diseases in environments with constrained laboratory infrastructure and resources. Therefore, they are crucial tools for positively impact the current state of healthcare in developing countries where accurate diagnosis of diseases is still a huge problem while making diagnosis faster in developed countries. These techniques have important advantages beyond molecular diagnosis in health facilities. They are being utilized in food safety to

Table 6
Shows example on applications strand displacement amplification in disease diagnosis.

Target	Sample	Template	Detection Method	Ref.
<i>Neisseria gonorrhea</i>	Genital and urine sample	DNA	Fluorescence probe.	[79]
<i>E. coli</i> 0157	Spiked serum	DNA	Gel electrophoresis, lateral flow assay.	[189]
<i>V. cholerae</i>	Spiked serum	DNA	Gel electrophoresis, lateral flow assay.	[189]
miRNA	Spiked Serum	miRNA	Gel electrophoresis & real time Fluorescence.	[190]
<i>S. aureus</i>	Nasal samples	DNA	Real time Fluorescence & lateral flow assay.	[82]
<i>Chlamydia trachomatis</i>	Genital and urine sample	DNA	Fluorescence probes.	[79]
Hepatitis	Serum	RNA	Gel electrophoresis & real time Fluorescence.	[191]
p53 gene detection	Serum	DNA	Molecular Beacon probe	[81]
Covid 19	Sputum	RNA	Fluorescence based	[186]

Table 7
Summary of isothermal amplification techniques comparison with PCR.

Feature	PCR	LAMP	RPA	SDA	HDA	NASBA	RCA	Ref.
Temperature (°C)	94–95,55–65,72	60-65	37-42	37- 42	37-65	40-65	23-60	[46,82,88, 89,91, 195–206]
Turn Around Time	1.5–3 h	30-65 min	20-40 min	Initial SDA up to 2 Hr. Modified SDA 15-30 MIN	60–90 min	1.5-2 Hr	~60min	
Enzyme/Protein	Taq DNA polymerase	Bst polymerase	DNA polymerase, recombinase, SSB	Restriction endonuclease <i>HincII</i> , exonuclease-deficient DNA polymerase.	DNA Polymerase, helicase &SSB	Reverse transcriptase, Rnase &T7 DNA-dependent RNA polymerase (DdRp),	Phi29 bacteriophage DNA polymerase, ligase.	
Detection	Gel electrophoresis, fluorescence, real-time	Fluorescence, turbidity gel electrophoresis, Lateral flow	Lateral flow, fluorescence, gel electrophoresis	Lateral flow, gel electrophoresis, fluorescence	Gel electrophoresis, fluorescence, Lateral flow	lateral flow fluorescence gel electrophoresis	lateral flow assay, gel electrophoresis. fluorescence	
Equipment	Thermocycler	Simple incubator/ water bath	Simple incubator	Simple incubator	Simple incubator	Simple incubator	Simple incubator	
Sensitivity (Limit of Detection)	~10 copies	~10 copies	~10 copies	>10 copies	~10 copies	~10 copies	~10 copies	
Efficiency	~10 ¹²	~10 ^{12 –13}	~10 ^{9 –10}	~10 ^{9 –10}	~10 ^{7 –9}	~10 ^{9–10¹¹}	~10 ^{10 –12}	

detect contaminants, in agriculture to detect plant pathogens and monitor crop health, and in environmental monitoring to track waterborne infections and many other fields. The reproducibility and amplification effectiveness of isothermal amplification techniques may be compromised by template integrity and deteriorated or contaminated materials. Assay reliability would be enhanced by using acceptable DNA or RNA concentration ranges, purity markers like A260/A280 ratios, and suggested sample preparation techniques. To make these techniques feasible in both economically and technically, several researches are being conducted globally. Further advancement is expected to improve its accuracy, broaden its uses, and better integrate it with digital technologies. With ongoing research and technological advancements, the potential of these technologies will significantly be enhanced in terms of sensitivity, specificity, and versatility, to positively impact global health, environmental safety, and various scientific fields.

CRedit authorship contribution statement

Alazar Amare Amdiyee: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Tesfaye Sisay Tessema:** Conceptualization, Validation, Writing – review & editing.

Funding statement

The authors received no specific funding for this work.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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