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Point-of-Care DNA Amplification for Disease Diagnosis and Management

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Abstract

Early detection of pests and pathogens is of paramount importance in reducing agricultural losses. One approach to early detection is point-of-care (POC) diagnostics, which can provide early warning and therefore allow fast deployment of preventive measures to slow down the establishment of crop diseases. Among the available diagnostic technologies, nucleic acid amplification–based diagnostics provide the highest sensitivity and specificity, and those technologies that forego the requirement for thermocycling show the most potential for use at POC. In this review, I discuss the progress, advantages, and disadvantages of the established and most promising POC amplification technologies. The success and usefulness of POC amplification are ultimately dependent on the availability of POC-friendly nucleic acid extraction methods and amplification readouts, which are also briefly discussed in the review.

1. INTRODUCTION

All available forecasts predict that the world population will continue growing at an exponential rate, but unfortunately no forecasts predict any increase in the Earth's area. By 2050, there will be 9.7 billion humans living on our planet (131), and agricultural scientists need to find ways to feed them in an ever more challenging environment with declining agricultural soil quality and climate change increasing unpredictability and extreme events. According to the Food and Agriculture Organization (FAO) of the United Nations, between 20 and 40% of global crop production is lost to diseases (110), with a cost of approximately USD $220 billion every year (31). An easy corollary is that we could increase global crop production 20–40% by just eliminating pests and diseases. Although this assertion is too simplistic, the truth is that approaches to disease management are far from optimal in developed countries and near chaotic in developing economies. In the absence of resistance, early pathogen detection and disease diagnosis in the field could be an invaluable asset for the efficiency of pest management programs and lessen crop losses.

The traditional methods to detect and identify plant pathogens are based on Koch's postulates, which have been an invaluable resource since their publication in 1876, but in today's technological era they have mostly outlived their usefulness. Symptom observation during field inspections is very useful, although many disease symptoms can be similar to those produced by other causes such as nutritional deficiencies and frequently need to be verified by clinical identification of the pathogen. Antibody-based methods provided a big advance in field-level diagnostics and have been widely and effectively used since the 1980s (3). Older technologies such as immunoblots and enzyme-linked immunosorbent assays are accurate and sensitive, although not ideally suited for field-based applications. Antibody-based lateral flow devices (LFDs) are more appropriate for point-of-care (POC), but their sensitivity is low compared to nucleic acid–based technologies. Other constraints of antibodies are that their production is costly and there can be inconsistencies in quality between batches.

The invention of the polymerase chain reaction (PCR) revolutionized biology in general and diagnostics in particular, as the method allowed the detection of pathogen-specific DNA sequences with great specificity and unprecedented sensitivity (63). Hundreds of PCR-based pathogen identification protocols have been developed for virtually all cultivated crops and most of the agriculturally important pathogens and pests. The advent of next-generation sequencing has also provided an invaluable resource for nucleic acid–based pathogen detection. The complete genomes of many pathogens are available in the public databases, allowing the use of bioinformatics to design primers for the detections of single pathogens, mutated variants, and, in a multiplex configuration, multiple pathogens in a single assay.

Nucleic acid–based POC disease diagnosis comprises three main steps: (*a*) on-site extraction and initial purification of nucleic acids; (*b*) amplification of the selected pathogen's nucleic acid with technology appropriate for use in the field; and (*c*) visualization of the results. To date, most public and private research efforts have centered on the development of simple, reliable, and efficient methods of nucleic acid amplification. Although not the focus of this review, consideration needs to be given to the first and third steps of the overall process. The initial extraction/preparation step is intimately connected to the success of the amplification protocol, as poor nucleic acid yield or excessive presence of inhibitors inevitably leads to an unacceptable level of false-negative results. In the same way, the visualization of results is also intrinsically linked to the amplification technology, as some methods are amenable to relatively simple visualization steps, whereas others require sophisticated equipment.

2. GENERAL CONSIDERATIONS

An eye towards practicality is essential for the development of useful POC diagnostic applications. Most importantly, ingenuity and creativity play a crucial role if the word "useful" is to be used next to "POC diagnostics." Many published POC diagnostic approaches are unrealistic and frequently demonstrate that the developers have not traveled for several hours to a remote field location and confronted the practical challenges faced in these environments.

When developing a POC diagnostic method, it is essential that the protocol can be performed in nonlaboratory, relatively dirty environments. Nevertheless, from years of experience performing diagnostic work in remote environments, I have realized that procedures do not necessarily need to occur in the open field. For example, the interior of a car can provide protection from the elements as well as a source of power. In this regard, our research team has developed simple, DC-powered devices to run loop-mediated isothermal amplification (LAMP) assays while parked or on the move (see Section 5). Alternatively, we have frequently collected samples from multiple locations and performed diagnostic procedures in a hotel room. Importantly, if the POC methodology is to be deployed by nonscientists, it is crucial to develop procedures that do not require extensive experience with molecular biology techniques and equipment. The incorrect use of a simple micropipette can ruin the most reliable and robust amplification-based diagnostic method.

3. POINT-OF-CARE SAMPLING

The goal of this step is to extract as much of the targeted pathogen's genomic material, either DNA or RNA, as possible while concurrently minimizing contaminants that could compete or interfere with the amplification reaction. But the goal of maximum genomic quantity and purity is also constrained by the need for technological simplicity while avoiding cross-contamination of samples. For example, the use of a mortar and pestle for grinding plant tissues is frequently impractical, and, instead, maceration of soft tissue in single-use Ziploc plastic bags with fingers or a metal rod allows for many samples to be processed quickly. Tougher plant tissues require methods such as shaking the sample in plastic tubes containing extraction buffer and metal ball bearings to achieve cellular disruption. Sampling from woody roots and tree trunks can be performed with a portable drill and 6–8 mm drill bits, which provide fine sawdust that easily releases cellular content into the extraction buffer. It is important to standardize the amount of tissue sampled to keep the buffer:tissue ratio as constant as possible. For this purpose, caps and lids are extremely useful. For example, closing an Eppendorf tube over a leaf excises an almost perfect circular tissue sample in a reproducible manner. Disposable caps from different sizes can be used to excise the appropriate amount of tissue or, in the case of wood shavings/sawdust, measure the initial tissue volume.

Impractical nucleic acid isolation methods include the traditional methods that utilize cetyltrimethylammonium bromide, guanidine thiocyanate, phenol/chloroform, or other toxic chemicals, as they are unsafe in remote environments away from medical assistance in case of accidents. Similarly, specialized commercial kits for nucleic acid purification, most of which use silica-based materials and chaotropic salts to bind nucleic acids and require multiple wash and centrifugation steps (12, 29), are frequently ill-suited for field conditions. In contrast, solid-phase reversible immobilization technologies based on paramagnetic beads coated with different chemical groups to bind nucleic acids are a step toward simplifying the extraction and purification process (9, 92). Immobilization of the paramagnetic beads to the side of the tube with a magnet eliminates the need for centrifugation after washes. Membrane-based extraction technologies such as lateral flow test (LFT) strips, Flinders Technology Associates cards (commonly known as FTA cards), and alumina membranes (56, 76, 119) bypass solvents and proceed directly to amplification

of nucleic acids bound to the membrane. Nonetheless, although simplicity has been gained, these methods can still be hindered by multiple pipetting steps (143) or require complicated experimental steps or electrical equipment not especially suited for in-field POC applications. For example, one published method (119) based on an LFT for nucleic acid purification requires cutting a 2-mm^2 square from the strip with a scalpel before adding it to the amplification tube.

A recent and more feasible approach to field-based DNA extraction uses a dipstick with a water-repellent handle and a small fragment (4 mm × 2 mm) of Whatman #1 paper at one end (143). Nucleic acid purification is achieved by immersing the dipstick in the tissue homogenate to bind nucleic acids before transfer to a tube containing a wash solution to eliminate contaminants and finally eluting the DNA directly into a tube containing amplification reagents. This method reduces the entire DNA purification time to less than 30 seconds and does not require any pipetting. Direct comparison experiments (143) showed that the dipstick outperformed a commercial paramagnetic bead kit and successfully purified amplification-ready DNA from multiple plant species infected with DNA- and RNA-based plant pathogens as well as human blood and melanoma cell lines. Dipsticks can be easily manufactured, and a detailed protocol has been recently published (86). The cost of this technology is almost negligible considering that one A4 sheet of Whatman #1 paper (210 mm × 297 mm) can produce in excess of 700 dipsticks.

4. POINT-OF-CARE NUCLEIC ACID AMPLIFICATION

4.1. Thermal Cycling Amplification

PCR, and its variants quantitative PCR (qPCR), reverse transcription-PCR (RT-PCR), etc., are unquestionably the most popular and best researched methods of nucleic acid amplification. There is ample documentation (49, 60) on the power, advantages, and disadvantages of PCR for diagnostic uses and therefore they are not discussed in this review. PCR uses thermal cycling at three different temperatures for the separation of the DNA strands, annealing of primers, and extension of the target sequence. Rapid heating and cooling of the samples is an energy-hungry process and not particularly suited for battery power supply. Nevertheless, rapid technological advances in power-efficient microelectronics and lithium-ion batteries have fueled the development of small, portable, battery-operated PCR and qPCR equipment. Portable machines rely on Bluetooth or Wi-Fi for communication and phone-based apps for control and visualization purposes, thus avoiding the need for LCD screens and other microprocessor-based components. Several commercial alternatives are available, such as the Franklin™ (**https://biomeme.com/**), which can process 9 samples on a battery that lasts up to 6 runs, and the Liberty16 (**https://www.ubiquitomebio.com/**), which accommodates up to 16 samples and can run on battery power for 2–3 hours. Although the new purpose-made amplification machines are amazing feats of miniaturization, weight is still an issue, as they range from 1 to 3 kg, and prices will never be able to compete with other less technologically demanding methods such as isothermal amplification (discussed in Section 4.2).

4.2. Isothermal Amplification

The ability to perform nucleic acid amplification at a single temperature eliminates the need for energy-demanding and technologically complex heating and cooling steps. Isothermal amplification methods have been available for more than 20 years and several different strategies have been developed to overcome the strand denaturation and primer annealing steps used by PCR. Although the list of existing isothermal amplification methods is now quite ample, many of them have been used only in a handful of publications; thus, we have a limited amount of knowledge about their optimal reaction parameters, design of primers, reproducibility, and efficiency and so

these are not included in this review. Some other technologies, despite being isothermal in nature, are practically complex and include numerous pipetting steps, making them unsuitable for POC applications. In this section, we discuss four of the most popular and promising isothermal amplification methods for in-field POC applications: LAMP, recombinase polymerase amplification (RPA), helicase-dependent amplification (HDA), and rolling circle amplification (RCA).

4.2.1. Loop-mediated isothermal amplification. LAMP is one of the oldest and best-established isothermal amplification methods; it was described for the first time in 2000 (94) and it has been widely adopted for multiple medical and agricultural applications (63, 67, 103, 130). LAMP uses a truncated DNA polymerase from *Bacillus stearothermophilus* (Bst polymerase) for DNA synthesis at an optimal temperature in the range of 60–65°C. The amplification process requires a minimum of four primers, two long inner primers [FIP (forward inner primer) and BIP (backward inner primer)] and two short outer primers (named F1 and B1), that recognize six specific sequences in the target DNA (94). The amplification of target sequences in LAMP is based on the initial synthesis of molecules containing stem-loop structures of single-stranded DNA (ssDNA) that can be used for priming and synthesis of longer products by the Bst strand displacement activity in a cycling manner; the process has been documented graphically in multiple reviews (e.g., 63). LAMP offers several advantages for POC applications such as its intrinsic speed, the use of a single temperature, stability of reagents at room temperature, capability to perform reverse transcription and amplification with a single enzyme, decreased sensitivity to amplification inhibitors, and wider working ranges in pH and temperature.

LAMP sensitivity has been repeatedly proved to be superior to conventional PCR, and different reports rank LAMP either slightly above or below the sensitivity of real-time qPCR and nested PCR (34, 43, 53, 127). Several improvements have been published to increase LAMP sensitivity, such as the use of two additional primers, known as loop primers, which can provide detection in half the time of the original LAMP (90). The addition of RecA recombinase to the reaction along with its required ATP substrate can also markedly increase efficiency and specificity (87), although later it was shown that this increase was due more to the ATP addition than the RecA addition (142). Moreover, increasing nucleotide concentration from the standard 4.8 mM to 8.8 mM resulted in a 16–20% reduction in detection time (142). Removal of betaine from the reaction mix can either further increase efficiency (37, 81) or have no effect (17, 140), and it has the drawback of increasing unspecific amplification with up to 33% of water controls producing an amplicon in some cases (142).

Further enhancements to LAMP sensitivity have been reported by adding supplementary swarm primers to the LAMP reaction mix (83). Swarm primers are added at relatively high concentrations targeting a region overlapping the F1/B1 sites and upstream of the FIP/BIP targets. The addition of swarm primers to conventional LAMP reactions increases the speed of the reaction and the final number of amplicons. Nevertheless, the effect of swarm primers seems to be dependent on the target template (142). Several reports have confirmed the efficiency of swarm primers, although at different scales, improving sensitivity 100 times in the detection of serotype O foot-and-mouth disease virus (74) and 10 times in the detection of porcine circovirus 3 (55); however, in the detection of *Fusarium oxysporum*, the beneficial effects were much less dramatic (142).

A marked advantage of LAMP for diagnostic assays is the fact that Bst has an innate reverse transcriptase activity, which allows the use of a single enzyme in the diagnosis of RNA viral pathogens. Although the reverse transcriptase activity of the original Bst enzyme was relatively low, new and improved versions of the enzyme, such as Bst 2.0 (**https://www.nebiolabs.com.au/products/m0537-bst-20-dna-polymerase#Product%20Information**) and Bst 3.0

(**https://www.nebiolabs.com.au/products/m0374-bst-3-0-dna-polymerase#Product%20Information**), have markedly improved reverse transcriptase activities with efficiencies comparable to RT-qPCR (46, 112). The addition of guanidine chloride can enhance LAMP sensitivity to levels comparable to RT-qPCR tests for the detection of viral pathogens such as SARS-CoV-2 (137).

Another advantage of LAMP, especially for POC applications, is the thermal stability of the reagents. LAMP reagents are stable at nonoptimal storage temperatures, with no observable decrease in efficiency reported when reagents were stored at 25°C or 37°C for up to 15 days compared to controls stored at −20°C (116). In addition, LAMP has a wider range of optimal pH and reaction temperatures than other amplification technologies. Direct comparison of qPCR and LAMP in diagnostic tests for the detection of *Salmonella enterica* serovar Typhi showed similar specificity and detection limits for both methods and LAMP proved to be more tolerant of variation in pH (7.3–9.3) and reaction temperature (57–67°C) (35). In POC situations, sample extraction and reaction preparation can cause delays, during which fully loaded amplification tubes can remain at room temperature for some time before incubation at the optimal temperature. These delays are unlikely to induce the generation of false positives in LAMP-based diagnostics, with incubation of fully loaded reactions for up to 10 minutes at room temperature before incubation at 65°C having minimal effect on amplification time (35, 142). In addition, engineered hot-start Bst variants such as Bst WarmStart® (**https://www.nebiolabs.com.au/products/m0538-bst-20-warmstart-dna-polymerase#Product%20Information**), which only allows amplification above a minimum temperature, are commercially available.

Despite the mentioned advantages of LAMP, this technology has limitations, which include the complexity of primer design. Several web-based algorithms are available for primer design such as Primer Explorer (**https://primerexplorer.jp/e/**) and the New England Biolabs LAMP primer design tool (**https://lamp.neb.com/**). Nevertheless, after years of experience, when we design a LAMP-based diagnostic method for a new pathogen, our protocol is to evaluate at least 8–10 different primer sets, of which typically one or two provide satisfactory performance. In addition, we have noticed that many published primer sets for specific pathogens do not perform as well as described in the original publication when reproduced in our laboratory, perhaps due to differences in the nucleic acid preparation process or other unknown and uncontrolled parameters. The use of nonoptimal primers can lead to multiple technical issues such as poor or no amplification of the template (false negatives) and generation of nonspecific amplicons in healthy samples and nontemplate controls by self-priming (false positives) (101). The generation of nonspecific amplification in LAMP reactions can be minimized by the inclusion of DMSO and betaine in the reaction (34, 126). Finally, as a consequence of the complex primer design, multiplexing is not an easy task for LAMP, although reports of multiplex detection are beginning to emerge (41, 136).

The extreme sensitivity of LAMP also brings the problem of carryover contamination from previous amplifications, resulting in false positives. A seemingly efficient solution to the carryover contamination problem has been reported by an ingenious combination of LAMP and CRISPR reagents (7). Typical FIP and BIP primers contain a linker between the 5′ and 3′ target homology regions. The so-called CUT-LAMP (contamination-free LAMP based on the CRISPR–Cas9 cleavage) introduces a protospacer adjacent motif (PAM) in the linker region of either FIP or BIP, resulting in the introduction of a cutting site for Cas9 with the proper guide RNA in all amplified products. Carryover contamination is avoided by including Cas9–gRNA ribonucleoprotein into the LAMP reaction mix and preincubating the reaction at 37°C for 5–10 mins followed by incubation at 65°C for the amplification of targets. Any previously amplified product is digested by the Cas9–gRNA ribonucleoprotein at the start of the reaction and does not produce amplicons, whereas genuine target DNA lacks the PAM and therefore is not affected by the preincubation (7).

Although compared to qPCR, LAMP can be less sensitive when used to quantify nucleic acid amount in a real-time context, combinations of LAMP–CRISPR and RT-LAMP–CRISPR platforms are emerging, mostly using Cas12a, Cas12b, or Cas13, with greatly increased sensitivity and specificity that also allow quantification of nucleic acids, although we are not aware of any use in plant diagnostics so far (2, 54, 72).

Specific and sensitive LAMP assays have been developed to detect multiple types of pathogens and pests in virtually all important food, fiber, and industrial crops, but because of space limitations only some are mentioned here (20, 28, 36, 39, 79, 115, 121). In rice, LAMP-based diagnostics have been developed for all the major fungal, bacterial, and viral diseases, including rice blast (caused by *Magnaporthe oryzae*), sheath blight (caused by *Rhizoctonia solani*), bacterial blight (caused by *Xanthomonas oryzae* pv. *oryzae*), bacterial leaf streak (caused by *Xanthomonas oryzae* pv. *oryzicola*), *Rice stripe virus*, and *Rice black-streaked dwarf virus* (14, 18, 61, 66, 71). Invasive pests such as nematodes in rice tissue or soil can also be efficiently detected (40, 134). LAMP-based diagnostics are available for the detection of devastating fungal pathogens in wheat, including *Puccinia triticina*, which causes wheat leaf rust (82), *Pyricularia oryzae*, which causes wheat blast (117), and *Fusarium graminearum*, which causes Fusarium head blight (1). Although Bst is by far the most popular enzyme used for LAMP amplification, screening of metagenomes in near-boiling pools has identified several thermostable polymerases with strong potential for nucleic acid amplification (104). One such screening discovered a PyroPhage 3173 DNA polymerase containing a proofreading 3′–5′ exonuclease domain that confers high fidelity in PCR (89). An engineered version of 3173 polymerase (later renamed as OmniAmp) was purified and used in RT-PCR reactions with sensitivity and specificity comparable to three different commercial two-enzyme systems (89). OmniAmp also has strong strand displacement capability and was optimized for use in LAMP and RT-LAMP diagnostic assays for multiple human and animal pathogens and outperformed a commercial Bst enzyme (15). OmniAmp exhibited several additional advantages such as lower sensitivity to inhibitors and higher stability compared to Taq and Bst polymerases. To the best of our knowledge, no reports are available on the use of this polymerase in plant diagnostics.

LAMP-based diagnostics also help protect national borders from new pathogens and pests by providing quarantine and inspection services with rapid POC systems (10, 114). Aside from plant pathogens, LAMP has important food safety applications, providing a convenient and fast method to detect health risks in food (58, 84, 93, 109). Finally, LAMP can be used to detect plant pathogens in potential vector carriers such as aphids and other insects, helping us to understand the transmission mechanisms for the disease and thus provide strategies for control or containment (21, 42, 66).

4.2.2. Recombinase polymerase amplification. RPA is similar to PCR, but it uses enzymatic means instead of temperature for DNA strand separation and primer annealing (97). Melting of the entire double-stranded DNA (dsDNA) template, as in PCR, is not required for primer binding, as unwinding of the corresponding short complementary regions is sufficient. The first step in the RPA process is the association of the forward and reverse primers with a recombinase, which helps them find the proper target and unwind a small region of dsDNA. After annealing, the structure is stabilized by the binding of a single-strand binding (SSB) protein, preventing primer ejection. Disassembly of the recombinase from the primers leaves their 3′ ends free and allows the start of transcription by the *Bacillus subtilis* (Bsu) Pol I DNA polymerase, which possesses strand displacement activity (97). To the best of our knowledge, RPA reagents can be obtained from a single commercial manufacturer, TwistDx (**https://www.twistdx.co.uk/**). Although PCR primers can be used in RPA reactions, the optimal primer length is 28–35 nt and the amplicon size is limited

to a maximum of 1,500 bp and preferably below 800 bp (97). According to manufacturer instructions, the RPA reaction can be performed at a temperature range of 30–42°C with an optimal temperature of 39°C.

RPA has several characteristics that make it an attractive amplification option for POC applications. First and foremost is its outstanding speed, with detection of 1–10 DNA target copies achieved within 20 minutes of the start of amplification, and it is not uncommon to detect amplification within 10 minutes (78). The low incubation temperature allows the use of simple battery-powered devices in the field, and there are reports of successful amplification using human body heat (22, 62, 133). RPA is amenable to multiplexing for the detection of multiple pathogens, although it is not as easy as with PCR (51, 62, 100). The simplicity of the primer design is a technical advantage over technologies such as LAMP, and like LAMP, RPA is relatively insensitive to common PCR inhibitors, providing an additional advantage for field POC applications where DNA purification might not be perfect (52). RPA components show extraordinary thermal stability, an essential characteristic for POC applications in remote locations or for the transport of samples to countries where a cold-storage supply chain is not available. RPA reagents stored at 25°C for up to 12 weeks did not show any decrease in amplification efficiency (no longer times were tested) and even storage at 45°C for 3 weeks did not result in loss of sensitivity (73).

A major drawback of RCA is the amplification of products due to a primer-dependent artifact inherent to the technology (97). When the amplification reaction is monitored by measuring the appearance of dsDNA by fluorescence intensity, using fluorescent-labeled primers or intercalating agents, the negative controls, either disease-free plants or water control, can produce an increased fluorescence intensity only a few minutes after the positive samples. In addition, the fluorescence intensity curves for the positive samples and controls are almost identical in shape, making it difficult to confidently assign the correct result to a sample (97, 142). In addition, in a field situation, the processing of multiple samples can be slow, resulting in initialized reactions sitting for some time at room temperature. Zou et al. (142) found that ten minutes preincubation at room temperature before heating to 37–39°C resulted in RCA fluorescence intensity plots that were indistinguishable between samples and controls (142). In addition, RPA primers can anneal to imperfect matches, with complex rules governing the effect of mismatches (23) and false positives resulting from amplifications with up to nine primer mismatches (13).

Several strategies avoid the problem of primer-mediated artifacts. First, the specific and nonspecific amplification products can be easily distinguished by electrophoresis analysis, as they have markedly different migration patterns. Similarly, the addition of a fluorophore-quencher probe and the double-strand-specific *Escherichia coli* endonuclease IV (Nfo) to the reaction mix can specifically target the positive amplification product (97). The probe consists of two oligonucleotides, containing either the fluorophore or quencher, which are linked by a tetrahydrofuran (THF) bridge, and it also contains a block at its 3′ end to prevent it from acting as a primer for amplification. Pairing of the probe to its complementary sequence in the target amplicon allows recognition of THF by Nfo, cleaving the probe and releasing the short 3′ oligonucleotide. Following the cleavage by Nfo, the fluorophore-quencher complex separates, producing a strong increase in fluorescence and leaving in place the remaining oligonucleotide with a free 3′ end that can serve as an additional amplification primer. The addition of the probe also increases the specificity of the reaction. Other strategies to avoid primer-mediated artifacts include the use of crowding agents to maximize amplification under normal circumstances, although they have a negative effect in low target copy number situations. When analyzing low copy number samples, it is important to either perform sporadic mixing or reduce the reaction volume (73).

The sensitivity of RPA for rapid nucleic acid detection can be enormously enhanced by coupling it with CRISPR-based technologies such as SHERLOCK, DETECTR, and iSCAN

(2, 16, 50), which have been optimized for the POC detection of crop viruses and fungal pathogens (4, 107). Although there are currently only a few examples, the potential of RPA–CRISPR hybrid technologies for POC applications is enormous. RPA has also been combined with other technologies such as surface-enhanced Raman scattering and electrochemical biosensors for the detection of *Botrytis cinerea*, *Pseudomonas syringae*, and *F. oxysporum* infections in plants (64, 65).

Commercial RPA-based diagnostic kits are available for several crop pathogens such as the *Banana bunchy top virus* and the devastating banana pathogen *F. oxysporum* f. sp. *vasinfectum*, Race 4 (Agdia, Elkhart, IN, USA). These kits can be used in field situations, although they still contain pipetting steps. Given that it is a newer technology, examples of diagnostic applications using RPA are fewer than for LAMP but are rapidly increasing, with applications reported for rice, maize, apple, and potato, among others (26, 47, 57, 138). RPA can be combined with an initial RT step to diagnose viroids and RNA viruses such as the tomato apical stunt viroid and the *Maize chlorotic mottle virus* (47, 59). Some of the examples mentioned here have been developed for POC applications, whereas others are mostly laboratory based. Nevertheless, when combined with an efficient nucleic acid isolation method and an appropriate POC readout, virtually all the described RPA-based methods can be optimized for POC applications.

4.2.3. Helicase-dependent amplification. The mechanistic principle for HDA is very simple: DNA unwinding is achieved with the help of a helicase enzyme and the ssDNA strands are stabilized by SSB proteins, allowing the forward and reverse primers to bind to initiate synthesis by the Klenow fragment of the DNA polymerase (123). The reaction proceeds at a single temperature (37°C) and requires several additional accessory proteins. After the first publication of HDA, a variation called thermophilic HDA was developed that utilized a thermostable helicase and Bst polymerase, which elevated the reaction temperature to 65°C and dispensed with the accessory proteins and other cofactors (5).

Even though simple in principle, HDA, like RPA, has a problem with nonspecific amplification arising from template-independent primer interactions (8). Control of nonspecific amplification can partially be achieved by the addition of betaine and DMSO, but it results in reduced amplification and loss of sensitivity (27). Aside from nonspecific amplification, an important drawback of HDA is the need for a lengthy optimization period to achieve an optimal equilibrium between the helicase DNA polymerase and reaction parameters. Amplification of long target regions by HDA can be problematic because of the low unwinding speed of the helicase. Although HDA-based diagnostics have been developed for clinical applications, there are very few reports of the use of HDA for plant diagnostics (105, 132).

4.2.4. Rolling circle amplification. RCA was the pioneer isothermal amplification technology (32). RCA uses ϕ29 DNA polymerase, which has strand displacement activity and used by itself produces a long single-stranded circular molecule of DNA from the position of a single annealed primer. To utilize RCA for diagnostic purposes, padlock probes are first utilized to prepare the sample for RCA. A padlock probe is a linear ssDNA molecule designed so that its 5′ (or 3′) ends hybridize with the target sequence in close proximity to adopt a circular conformation similar to a padlock (63). After hybridization, the probe molecule is ligated with T4 ligase to form a covalently linked ssDNA circular molecule. RCA is then performed with a primer that anneals to the newly formed circular ssDNA molecule. This protocol is readily multiplexed and has been used for the diagnosis of plant diseases, although the multiple hybridization and enzymatic steps involved in the formation of the padlock and RCA amplification make it too labor intensive for POC applications (108, 111, 118).

4.2.5. Other isothermal amplification methods. Other isothermal amplification methods have been developed but have found limited adoption in plant diagnostics for several reasons, including cost and relative complexity. These methods include nucleic acid sequence–based amplification (19), strand displacement amplification (124), cross-priming amplification (30), isothermal chain amplification (48), polymerase spiral reaction (PSR) (77), strand exchange amplification (SEA) (106), and CRISPR-Cas9-triggered nicking endonuclease–mediated strand displacement amplification (141).

Generally, lesser-known isothermal amplification techniques have poorly established rules for primer design and reaction optimization. A recent study compared five isothermal amplification techniques—SEA, PSR, CPA, RPA, and LAMP—to detect the *F. oxysporum* endopolygalacturonase gene (142). PSR generally failed to amplify the target even though eight different primer sets were designed following instructions from the original publication. Similarly, three different primer sets designed as described in the original publication for CPA either failed to produce an amplicon or produced amplification in the water controls, although a modification of the original primer design rules achieved reliable CPA-mediated amplification. Establishment of the detection limits for these isothermal techniques revealed that only LAMP and RPA provided sufficient sensitivity for diagnostic purposes (142).

5. POINT-OF-CARE AMPLIFICATION READOUTS

Verification of the success, or lack thereof, of the amplification reaction is the final key to efficient pathogen detection in a POC setting. Monitoring is achieved by two approaches: either by direct detection of newly synthesized DNA or indirectly by measuring changes in the reaction solution. Intercalating dyes such as SYBR Green I, EvaGreen, and the large family of cyanine SYTO dyes bind to dsDNA, dramatically increasing the intensity of their native fluorescence and making them useful for rapid detection of amplicons. Detection of intercalating agents can be achieved with electronic detectors or by the naked eye. SYBR Green I was one of the first commercially available dyes and has been extensively used for qPCR reactions in thermocyclers (69). SYBR Green I and EvaGreen have been successfully used to monitor LAMP reactions using electronic equipment, although the increased reagent concentration required for naked-eye detection resulted in strong inhibition of the reaction (33). An alternative is to add the DNA-binding dyes after the amplification process, but the process of opening the reaction tube increases the risk of cross-contamination of samples, especially in nonoptimal POC environments. Many intercalating dyes have a weak but measurable affinity for ssDNA, thus introducing background noise. A comparative study of six fluorescent intercalating dyes (SYTO-9, SYTO-13, SYTO-82, SYBR Green I, SYBR Gold, EvaGreen) using electronic detection identified SYTO-82 as the best reagent for LAMP assays, offering the best combination of amplification speed and signal-to-noise ratio (95).

In contrast to direct measurement of amplified DNA, reaction by-products can also be used to determine amplification success. LAMP reactions produce a reasonable amount of insoluble magnesium pyrophosphate, which remains suspended in the amplification reaction, increasing turbidity (88). The increase in turbidity is proportional to the amount of DNA synthesized and can be used as an amplification readout. The spectrophotometric measure of light scattering caused by turbidity has been used as an accurate measure of amplification (88). Nevertheless, the use of the naked eye to detect turbidity needs an experienced operator, and turbidity is observable only in strong amplifications (88). The depletion of soluble magnesium during LAMP amplification can also be monitored with hydroxy naphthol blue (HNB), which shifts from violet to sky blue as soluble Mg is exhausted (38). In contrast to SYBR Green I, HNB does not interfere with the amplification and therefore can be added at the start, which decreases the risk of cross-contamination.

Although the color difference between a control and a positive reaction can be easy to observe, slight color shifts in reactions with partial magnesium consumption are difficult to evaluate with the naked eye (38). pH color indicators have also been used to measure pH changes due to the release of H^+ ions during DNA synthesis, although for this strategy to be effective it is necessary to use weak buffers, which introduces additional risks because imperfectly prepared samples in POC situations could produce pH changes affecting the efficiency of the enzymatic reaction (113).

Although color change is in principle an easy indication of amplification success, human eye interpretation of color and color intensity can vary markedly between individual operators, providing a source of error in data interpretation. Moreover, even with the same operator, color hue and intensity can be interpreted in markedly different ways depending on the external light conditions; e.g., sunny versus cloudy days or mornings versus late afternoons (96, 128). It is therefore advisable to avoid human interpretation in systems generating a continuous color output.

Nanoparticle aggregation-based methods, including the use of gold nanoparticles (GNPs) with covalently attached probes, have also been developed for the detection of successful amplifications (122). Alternatively, aggregation of paramagnetic particles has been used as a naked-eye indicator of successful amplification. dsDNA can be visualized by the degree of particle aggregation after mixing the final reaction products and paramagnetic particles in a rotating magnetic field (70). Two similar strategies also used aggregation of paramagnetic particles without the need for rotating magnetic fields, although these protocols required multiple pipetting steps and thus are not very amenable to POC field conditions (75, 129). A recent report used a mix of charcoal and diatomaceous earth particles in suspension, which quickly precipitates by bridging flocculation in the presence of dsDNA without any manipulation, providing a black/transparent, easy-to-see readout in a few seconds (85). All these approaches require the opening of the tube containing final amplification products, with the consequent increase in cross-contamination risks, as discussed above.

A completely different approach uses lateral flow assays (LFAs) as amplification readouts (139). LFAs have been used for more than 60 years and offer a unique combination of speed, relatively low cost, and simplicity with a clear yes/no readout that makes them ideal for POC use. They have become extremely popular in scientific and medical applications, with pregnancy tests, arguably the most popular LFA, available in many supermarkets. Initially, LFAs detected peptides and proteins with specific antibodies, but have evolved to detect many other biological molecules, including DNA (139). In a typical LFD, samples are applied to the absorbing pad at one end, and as the sample migrates by capillarity, the target molecule is recognized by specific antibodies (Ab1) conjugated to GNPs in the conjugate pad. The target-Ab1-GNP complex continues its migration until reaching the test line, where it binds a second immobilized target-specific antibody (Ab2) recognizing a different epitope in the target, making a "sandwich" Ab2-target-Ab1-GNP. The accumulation of GNPs in the test line results in an easy-to-see colored band in positive tests. Beyond the test line, a control zone containing immobilized anti-IgG antibodies captures the excess Ab1-GNP conjugates and provides a positive control for the LFA (6). A modification of LFA for nucleic acid detection comprises the use of forward and reverse primers labeled at their 5′ ends with biotin and fluorescein amidite (FAM), respectively; in the case of LAMP, the labels are bound to the FIP and BIP primers. After amplification, the newly generated dsDNA amplicons contain both labels incorporated into their structures. For detection of amplicons, the conjugate pad contains anti-FAM antibodies conjugated to GNPs (FAM-Ab-GNP), whereas the test zone contains immobilized streptavidin. Biotin- and FAM-containing amplicons diffusing along the LFD bind the FAM-Ab-GNP complexes in the conjugate zone and continue migrating to the test zone, where the advance of the amplicons is stopped by binding of the biotin groups to the membrane-bound streptavidin; the result is a red band in the LFD (an excellent graphic explanation can

be found in Reference 139). Biotinylated and FAM-labeled oligonucleotides are inexpensive, and LFDs for the detection of biotin/FAM-labeled amplicons are readily available from several commercial suppliers, making the implementation of this approach straightforward.

Nucleic acid–based LFAs can be less sensitive than direct detection of amplicons in assay tubes. For example, RPA-LFA methods were 10 times less sensitive than qPCR for the detection of potato virus X, whereas LAMP-LFA methods showed similar sensitivity to PCR (45). LFAs are amenable to the detection and discrimination of different amplicons in multiplex amplification reactions (139). As above, a disadvantage of amplification-LFA approaches is the need to open the amplification assay tube for loading of the LFD, increasing the risk of cross-contamination.

LFAs in combination with isothermal amplification have been widely used in plant diagnostics. RPA-LFA methods have been developed in multiple crops, including potato, pepper, soy, strawberry, and citrus, for the detection of viral, bacterial, and phytoplasma pathogens (11, 24, 25, 44, 68, 80, 125, 135). An LFA-RPA multiplex system has been developed for detection of the genus *Clavibacter* in maize (62). Although less abundant than RPA, LAMP-LFA methods are available

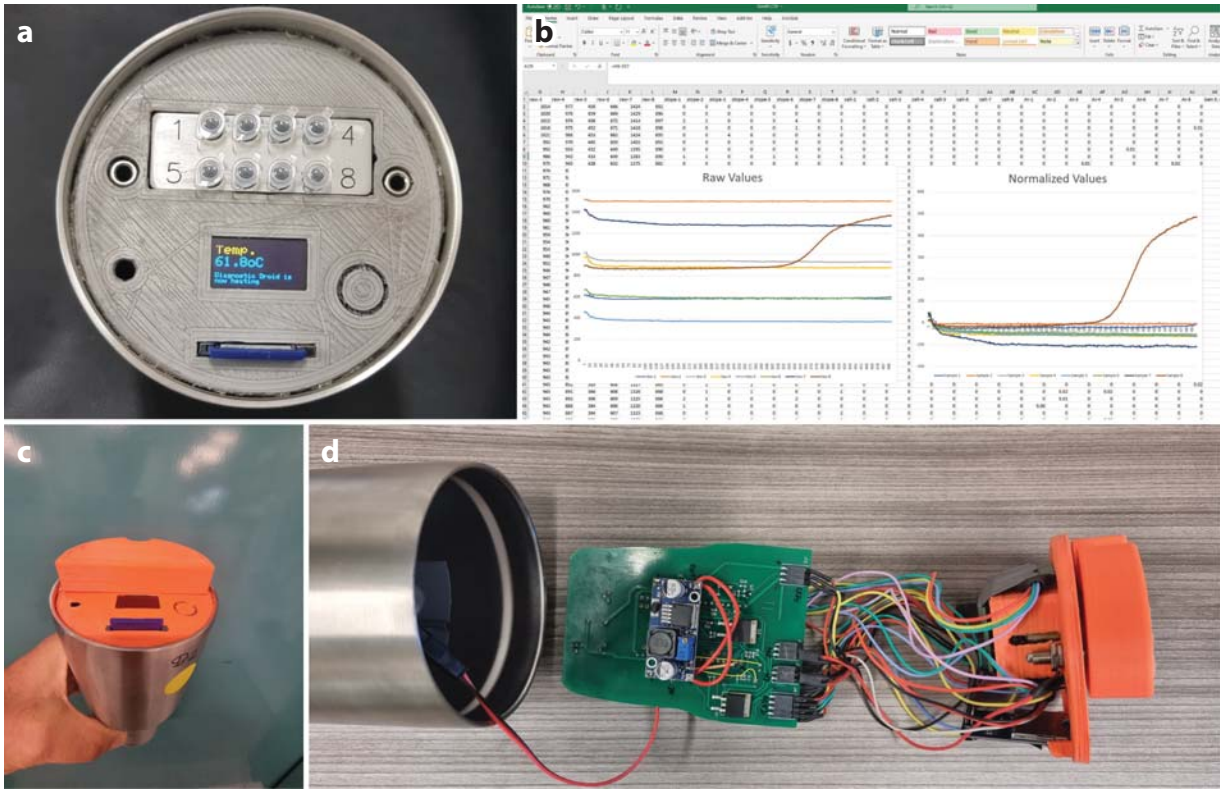

Figure 1

Real-time loop-mediated isothermal amplification (LAMP) hand-held device. Diagnostic Droid. (*a*) Top view of a Diagnostic Droid showing the heating block loaded with eight LAMP reaction tubes. LED screen shows the block temperature. Once the reaction starts, the LED screen displays the results of the reaction (positive/negative/inconclusive) for each tube in real time. Data are automatically recorded on an SD card (*seen in the photo*). (*b*) Data are collected on the SD memory card in an Excel file and can be easily plotted using either raw values from the light detectors or normalized values. (*c*) Diagnostic Droid with the heated lid in place. Plastic components are made using 3D printing. (*d*) Components of the Diagnostic Droid. The printed circuit board fits inside the coffee mug and connections are made with the LEDs, sensors, SD card, and LED screen.

for the detection of viral, fungal, bacterial, and phytoplasma pathogens in multiple plants, including tobacco, potato, sugarcane, and citrus (91, 98, 99, 102, 120). Multiplex LAMP-LFA methods are also available, including the simultaneous detection of *Tobacco rattle virus* and *Potato virus X* in potato (28).

Electronic equipment to incubate the amplification reaction, monitor the development of the amplification, and visualize the results does not necessarily need to be complex, large, or expensive. Our research team has designed and built a relatively simple POC device, named the Diagnostic Droid, using an Arduino-based platform, readily available LEDs, and light sensors to incubate and monitor LAMP amplification reactions in the field (**Figure 1**). Up to eight tubes are incubated in a metal block at 65°C with side illumination by the LEDs while the sensors placed at the bottom measure the intensity of scattered light caused by the increase of turbidity upon the generation of insoluble magnesium pyrophosphate. Turbidity is measured at regular intervals and the values are interpreted by a mathematic algorithm that detects logarithmic increases in turbidity, providing a positive/negative result easy to interpret by nonspecialist personnel. The Diagnostic Droid is contained within a metallic thermal coffee mug for protection and easy transport in a car drink holder and can be powered from the car lighter socket or USB power supply. An LED screen provides instant results (tube number, positive/negative/inconclusive), and data are recorded on an SD memory card for further analysis if required.

6. CONCLUSIONS

It is important to carefully think about the intended use and, most importantly, the specific circumstances in which the diagnostics will be performed before choosing a POC amplification technology. Parameters to consider are the nature of the pathogen genome (DNA versus RNA); the degree of specificity needed for the amplification, determined by the specific species, bacterial pathovar, fungal strain, etc.; and the assay tissue choice, determined by the biology of the pathogen. POC-specific parameters such as the expected climate, availability of electrical supply, geographical location, and availability of local commercial suppliers for amplification reagents also influence the choice of POC amplification.

From the available amplification technologies, the isothermal LAMP and RPA provide the best systems for POC amplification of plant pathogens and numerous examples are available in the literature for plant-pathogen detection and identification. Although LAMP has been the preferred method of choice for POC applications for a long time, RPA is quickly catching up and might become the preferred method in the future. Whatever the amplification method, it needs to be considered in conjunction with an appropriate sampling and nucleic extraction/purification method, minimization of contamination risk, and a compatible readout to visualize/interpret results.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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