NUCLEIC ACID-BASED METHODS FOR ON-SITE DETECTION OF PLANT PATHOGENS: APPROACHES AND APPLICATIONS

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Thesis submitted to the University of Nottingham
for the degree of Doctor of Philosophy

DECEMBER 2012

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ABSTRACT

The ability to perform nucleic acid-based detection of plant pathogens away from conventional laboratory facilities has the potential to be beneficial in situations where results are required very rapidly or where resources and access to laboratory equipment are limited. Methods for use in such situations must combine sensitivity and specificity with rapid and simple workflows. The aim of this project was to investigate aspects of on-site testing for plant pathogens by developing detection methods for a range of target species.

Detection methods based on loop-mediated isothermal amplification (LAMP) exhibit characteristics which make them potentially suitable for on-site testing. LAMP-based methods were developed for detection of plant pathogens with three potential non-laboratory testing scenarios in mind: testing during plant health inspection (assays for *Phytophthora ramorum*, *P. kernoviae* and *Guignardia citricarpa*); testing to assess inoculum levels in the processing of plant products (an assay for *Botrytis cinerea*); and testing in under-resourced settings (assays for *Cassava brown streak virus* and *Ugandan cassava brown streak virus*). In developing these detection methods, attempts were made to address some of the specific requirements of potential end-users of the tests in each case.

For testing in the context of inspection, a particular emphasis was placed on the need for simple, rapid methods for nucleic acid extraction. As well as investigating the use of rapid extraction methods in conjunction with LAMP, work was also carried out to investigate how on-site nucleic acid extraction using lateral flow devices could be integrated with current field and laboratory testing for *P. ramorum*.

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ACKNOWLEDGEMENTS

I would like to acknowledge my supervisors, Neil Boonham and Matt Dickinson; I am extremely grateful for their support and encouragement and for the opportunities they have given me.

I would also like to thank my colleagues at the Food and Environment Research Agency for their assistance and for the generosity with which they share their expertise. In particular, I would like to thank Jennifer Hodgetts and Ian Adams for their advice and support during the preparation of this thesis, and Sioban Ostoja-Starzewska and Catherine Harrison, with whom it is my on-going pleasure to work.

And Pete, Alice and Ella, my mum Linda, my dad John and my grandparents: thanks for everything.

CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

bp	base pairs
CFU	colony forming unit
COX	cytochrome oxidase gene
CPA	carrot piece agar
CTAB	cetyltrimethylammonium bromide
Ct	Cycle threshold
Defra	Department for the Environment, Food and Rural Affairs
DIG	digoxigenin
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbant assay
EPPO	European and Mediterranean Plant Protection Organisation
Fera	Food and Environment Research Agency
FITC	fluorescein isothiocyanate
HDA	helicase-dependent amplification
HNB	hydroxy naphthol blue
IGS	intergenic spacer
ITS	internal transcribed spacer
LAMP	loop-mediated isothermal amplification
LFD	lateral flow device
NALF	nucleic acid lateral flow
NASBA	nucleic acid sequence-based amplification
NPPO	national plant protection organisation
NPV	negative predictive value
PCR	polymerase chain reaction
PDA	potato dextrose agar
PPV	positive predictive value
rDNA / rRNA	ribosomal DNA / ribosomal RNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RPA	recombinase polymerase amplification
RPPO	regional plant protection organisation

RT-LAMP	reverse transcription loop-mediated isothermal amplification
RT-PCR	reverse transcription polymerase chain reaction
Tp	time to positive

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CHAPTER 1 INTRODUCTION

1.1 Applications for plant pathogen detection

Methods for the accurate detection of plant pathogens and pests are valuable tools in a range of contexts, including research into pathogen biology and epidemiology. Beyond use in the study of plant pathology, methods for detection of pathogens are fundamental to much of the work carried out by national plant protection organisations (NPPOs) (Miller et al. 2009). A major application for accurate detection methods is in the context of surveillance for quarantine pests and pathogens, for example, in imported plants and plant products. Methods for specific detection of organisms can also be used as diagnostic tools to determine the presence or absence of pathogens which could be causing the symptoms of disease, allowing appropriate disease control measures to be taken. In these contexts, the efficient deployment of accurate diagnostic tools serves the primary purpose of aiding and expediting decision making to better manage disease or to prevent the introduction and spread of quarantine pathogens.

Regardless of the application, detection methods must be sensitive and specific enough to provide useful information to the end-user. For certain applications, another critical factor is the speed with which results can be obtained. In order to generate results in a timely manner in the laboratory, efforts can be made to maximise throughput and efficiency, often with the introduction of automated or semi-automated testing. Beyond this, however, there are some applications where significant advantages could be gained by moving testing to the site of sampling, avoiding the need to send to samples to a centralised laboratory facility. Examples of such applications include import

inspection of perishable commodities for quarantine pathogens and pests, or testing for storage pathogens in food production or the supply chain. On-site testing could alternatively be used as a primary screen to reduce the number of samples submitted to the laboratory for testing. Furthermore, methods which are found to be suitable for these non-laboratory applications also have the potential to be deployed in settings where testing is currently restricted by limitations of facilities or resources, for example, in developing countries. Methods for on-site detection of plant pathogens therefore have the potential to be used to achieve two main objectives: to expedite decisions regarding disease control and management in the context of inspection or industry, and to allow testing to be performed in settings where the use of conventional laboratory methods is not possible.

1.2 Approaches used for the detection of plant pathogens

Plant pathogens can be detected using a variety of approaches, many of which involve direct observation of a pathogen or its effects on the plant, for example, assessment of symptoms, microscopy, culturing and biological assays including bait tests and inoculation onto indicator plants. Diseased plants may also be detected by remote sensing using imaging (Sankaran et al. 2010) or acoustic detection of pest activity (Mankin et al. 2011). Antibody-based detection methods are well established and are used for routine detection of various pathogens (Danks and Barker 2000; Ward et al. 2004). Monoclonal antibodies can be developed for detection of target-specific antigens (Werres and Steffens 1994; Ward et al. 2004) and incorporated into laboratory tests, such as enzyme-linked immunosorbant assay (ELISA), or lateral flow devices

(LFDs) suitable for field use. However, development of monoclonal antibodies can be time consuming, and in some cases the antibodies that are developed may display insufficient sensitivity or specificity, or may not reliably detect all life stages (Ward et al. 2004). Further difficulty may be encountered in the detection of low-titre pathogens, for example, in asymptomatic or latent infection. Nevertheless, ELISA is widely deployed for routine detection of some targets, including many plant pathogenic viruses, and immunoassays in LFD formats were among the first tests to enable routine testing for plant pathogens in the field (Danks and Barker 2000; Lane et al. 2007). The most common approach to testing for pathogens for which specific antibodies are not available, or where a higher sensitivity or specificity is required, is the use of nucleic acid-based detection.

Methods for detection of nucleic acid targets are typically more sensitive than serological tests. Furthermore, if relevant sequence data is available, specificity can be manipulated to target the taxonomic grouping that is most relevant to disease, which may be at the level of species, sub-species or pathovar. Development of nucleic acid-based methods can also typically be completed more quickly than development of new antibodies. Methods based on the polymerase chain reaction (PCR) are well established for numerous applications in molecular biology. Conventional PCR and reverse transcription-PCR (RT-PCR) requires post-amplification analysis, usually by gel electrophoresis of amplification products. This is relatively laborious, and as a result the use of conventional PCR for routine plant pathogen detection is fairly limited (Mumford et al. 2006). Many smaller laboratories favour ELISA or culturing, with PCR-based detection used mainly for pathogens for which

testing by one or both of these methods is not possible (for example, unculturable pathogens including phytoplasmas and viroids). Many of the disadvantages of conventional PCR are addressed by the use of real-time PCR, in which the generation or accumulation of amplification products is monitored in real-time, obviating the need for laborious post-amplification processing. Real-time monitoring of PCR is achieved through fluorescence detection, with the use of either a fluorescent probe or an intercalating dye such as SYBR Green. Various real-time PCR chemistries have been developed (Wong and Medrano 2005), but TaqMan probes and SYBR Green are the most commonly used for detection of plant pathogens (Mumford et al. 2006). Target sequences for real-time PCR are typically short (60 to 120 bases), allowing faster thermal cycling to be used for real-time PCR than for conventional PCR. In combination with the lack of post-amplification manipulations, this allows real-time PCR to be completed in less than 2 hours, while conventional PCR followed by gel electrophoresis typically takes around 3 to 5 hours. The sensitivity of fluorescence detection and the additional specificity conferred by the probe in some formats of real-time PCR result in levels of analytical sensitivity and specificity that typically exceed those of comparable conventional PCR assays (e.g., Korimbocus et al. 2002). The practical advantages and performance characteristics of real-time PCR have led to the adoption of this method for routine testing for a wider range of plant pathogens than conventional PCR and for applications where pathogen titre prevents the use of ELISA, such as direct tuber testing for potato viruses (Mumford et al. 2006; Boonham et al. 2008). The major drawback of real-time PCR is the cost in comparison with culturing and serological methods. In particular, real-time

PCR platforms for simultaneous thermal cycling and fluorescence detection are complex and relatively costly (typically in excess of £25,000 for an entry-level instrument). Recent advances in real-time PCR instrumentation and technologies have sought to increase throughput and automation; for example, real-time PCR arrays allow thousands of reactions to be performed in parallel (Morrison et al. 2006; Spurgeon et al.; 2008 van Doorn et al. 2009). However, these developments only add to the complexity and cost of the instrumentation required. In general, the use of real-time PCR is therefore accessible only to well-equipped centralised facilities.

1.3 On-site testing for plant pathogens

Due to the potential benefits of moving testing closer to the point of sampling, efforts have been made to develop methods for on-site testing which have performance characteristics which approximate those of nucleic acid-based testing in the laboratory. Since high-performance detection in the laboratory has increasingly been achieved by real-time PCR, initial attempts were made to transfer real-time PCR from the laboratory to the field (Schaad et al. 2002; Mavrodieva et al. 2004; Tomlinson et al. 2005). The major factor in allowing real-time PCR to be carried out in non-laboratory conditions was the availability of portable and ruggedised real-time PCR platforms (Mumford et al. 2006). Due to the requirement for rapid and accurate thermal cycling with concurrent fluorescence detection, these instruments are nevertheless complex and relatively costly, despite being smaller and more portable than laboratory-based machines. Another significant issue for successful on-site use of real-time PCR is the requirement for nucleic acid extraction methods which can be

used in the field. Extraction protocols based on the manipulation of magnetic beads were modified to minimise or eliminate requirements for laboratory equipment, but these methods still involved multiple manipulations (Tomlinson et al. 2005). In essence, the methods developed for on-site nucleic acid extraction and real-time PCR comprised modifications of inherently laboratory-based techniques and were not widely adopted, although on-site real-time PCR using the Cepheid SmartCycler has been successfully deployed for plant pathogen detection in the UK for a small number of applications including testing for *Phytophthora ramorum* at outbreak sites (Hughes et al. 2006a). In order to develop on-site nucleic acid-based detection methods with broader applicability, a potentially more fruitful approach is to identify technologies for nucleic acid extraction and amplification that are specifically suited to on-site testing, rather than modifying established laboratory techniques.

1.4 Approaches to nucleic acid extraction for on-site detection

Criteria by which extraction methods are evaluated typically include nucleic acid yield and purity. To achieve reliable detection by PCR-based methods, it is generally necessary to test high-quality nucleic acid extracts, and this requires the use of methods that effectively remove substances which are inhibitory to PCR. Plant material and other matrices relevant to the detection of plant pathogens, such as soil, contain substances including acidic polysaccharides and polyphenols which are known to inhibit PCR and which must be removed during nucleic acid extraction, or otherwise PCR additives used to counteract their effects (Wilson 1997; Ikeda et al. 2008). As well as

purity and yield, however, a significant factor in developing extraction methods specifically for on-site use is the need for the workflow to be rapid and simple, with as few manipulations as possible (Mumford et al. 2006).

The Food and Environment Research Agency has recently developed a method for extracting nucleic acid from matrices including plant material using LFD membranes (Danks and Boonham 2007; Tomlinson et al. 2010a). In this method, samples are disrupted in an extraction buffer (for example, by shaking with ball-bearings) and applied to an LFD, after which a portion of the nitrocellulose membrane of the device is excised and added to a nucleic acid amplification reaction (such as PCR), as shown in Figure 1.1. This method is sufficiently simple to use in the field, as the workflow is identical to that used for immunoassays in LFD format. Furthermore, the nucleic acid is stable on the LFD membrane at room temperature, allowing extraction to be performed in the field and the devices sent to the laboratory for testing, avoiding transportation of potentially infected plant material.

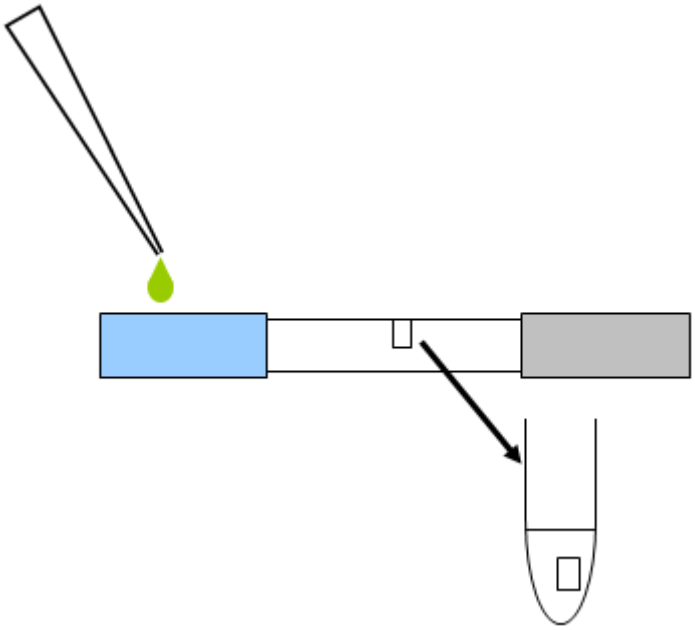

Figure 1.1 Nucleic acid extraction using a lateral flow device, as an example of a method suitable for on-site use. The method consists of only three manipulations: (i) manual disruption of the sample in buffer (for example, by shaking with ball bearings); (ii) application of the sample to the device; (iii) excision of a section of the membrane for testing by nucleic acid amplification.

Other methods have been developed for stabilisation of nucleic acids in the field for later testing (Roy and Nassuth, 2005), although these generally require some additional processing before the nucleic acid can be amplified. Fukasawa et al. (2010) recently described a rapid method for purifying DNA by filtration following the formation of DNA-Mg^{2+} complexes under alkaline conditions, which could be applicable to on-site use. Such methods share key features of being significantly faster than conventional extraction methods and requiring little or no laboratory equipment. Simplicity of workflow is particularly critical for methods to be deployed by non-specialist end-users. Minimising the number of manipulations required reduces the chance of contamination or other errors. Furthermore, simple and rapid methods can be more readily incorporated into existing processes (for example, inspection or quality control activities) and are therefore more likely to be adopted than more complex and time consuming methods.

1.5 Isothermal amplification of nucleic acid for on-site testing

The majority of nucleic acid detection methods in current use employ the paradigm of detection by amplification, whereby a target-specific sequence is identified and a reaction is devised to amplify this target to levels greatly exceeding the background, allowing it to be detected. Other approaches exist, for example, detection of double-stranded DNA (Ghosh et al. 2006) and use of various biosensors (Craw and Balachandran 2012), but amplification is currently by far the most common method for detection of specific nucleic acid sequences. Isothermal amplification methods have been developed which circumvent the major disadvantage of PCR for non-laboratory use, namely the

requirement for complex thermal cycling equipment, and some of these methods have the potential to be used outside the laboratory.

Isothermal amplification methods share with PCR the central concept of the extension of target-specific primers by DNA polymerase (or in some cases, RNA polymerase). In general terms, the challenge of isothermal amplification is therefore to enable primer binding, such that amplification can occur without the repeated cycles of denaturation and annealing required for PCR. There are a number of approaches to the generation of single-stranded primer binding sites without thermal cycling, including methods based on non-thermal methods of template denaturation, transcription of RNA, strand displacement around a circular template, nicking or partial degradation of primer extension products to allow extension or further rounds of priming, and formation of secondary structure containing single-stranded primer binding sites. Methods for isothermal amplification described to date have been reviewed in detail (Gill and Ghaemi, 2008; Asiello and Baeumner 2011; Niemz et al. 2011; Craw and Balachandran 2012), and range from those which are relatively well established to those which are in the early stages of development. Nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP) are the most well established methods for isothermal amplification of nucleic acids to date, and detection of various plant pathogens has been demonstrated using each of these chemistries. Plant pathogen detection has also been demonstrated using rolling circle amplification (RCA) and isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN), although these methods are less common. Other recently developed isothermal amplification chemistries

displaying characteristics which could be beneficial in a field testing context include helicase-dependent amplification (HDA) and recombinase polymerase amplification (RPA).

1.5.1 Helicase-dependent amplification and recombinase polymerase amplification

A conceptually simple approach to achieving isothermal amplification of DNA is to separate the strands of the double-stranded template by non-thermal means. HDA (Vincent et al. 2004) and RPA (Piepenburg et al. 2006) are two examples of this approach. HDA uses a helicase to separate the strands of double-stranded DNA allowing primer binding and extension by DNA polymerase at a constant temperature of approximately 65°C. Reaction times for HDA are generally in the range 30 to 90 minutes. This method sustains amplification of relatively short products of approximately 70 to 120 bp (Andresen et al. 2009), although a variant of HDA has been described using a novel enzyme with combined helicase and polymerase activity which can generate longer amplicons (Motré et al. 2008). HDA can be performed at a single temperature, but the inclusion of a brief incubation at 95°C prior to the addition of the HDA enzymes can increase sensitivity.

RPA uses recombinase which forms a complex with primers to initiate amplification without thermal denaturation (Piepenburg et al. 2006). RPA does not require an initial denaturation step and has a low reaction temperature (between 37 and 42ºC) which can easily be sustained by a low power instrument. However, the use of a low reaction temperature can result in the generation of more non-specific amplification artefacts than are typically

observed in isothermal amplification methods which use higher reaction temperatures. Primer design for RPA is subject to additional constraints to those for design of primers for PCR and other amplification methods, due to the requirement for primers that interact optimally with recombinase. For this reason, and because the low reaction temperature requires that the primers should not form homo- or heterodimers that could result in the generation of artefacts, RPA assay development can require a heuristic approach to be taken as RPA primer design software is not yet available. A portable platform for real-time RPA is commercially available (TwistDx). The major advantage of RPA is its short reaction times, which are typically <30 minutes (Piepenburg et al. 2006). While conceptually simple, the reaction components of both HDA and RPA are relatively complex, such that development of assays has depended on the use of reagent kits which have only recently become commercially available. The advantages and disadvantages of these methods, and their applicability to different targets including plant pathogens, will become apparent as they become more established.

1.5.2 Nucleic acid sequence-based amplification

Nucleic acid sequence-based amplification (NASBA) is a method for isothermal amplification of RNA based on transcription (Compton, 1991). A modified primer is used to incorporate the sequence of the T7 RNA polymerase promoter into a double-stranded DNA intermediate, functionalising the promoter and resulting in transcription of a single-stranded RNA product at a reaction temperature of 41ºC. The single-stranded nature of the amplification product makes NASBA particularly suited to hybridisation-

based detection methods (Bentsink et al. 2002; Olmos et al. 2007). NASBA has been used for the detection of a number of plant pathogens in conjunction with molecular beacon probes, in a format sometimes referred to as AmpliDet (Leone et al. 1998; Klerks et al. 2001; Van der Wolf et al. 2004). This format, in which fluorescence is monitored in real time to detect hybridisation of the probe to the single-stranded amplicon, is a closed-tube system and allows quantification of the target sequence, but requires the use of an instrument with real-time fluorescence monitoring capability. A notable feature of NASBA is the inherent RNA-selectivity of the amplification mechanism. Because mRNA is less stable than DNA and degrades rapidly in dead cells, this enables NASBA to be used to specifically detect viable cells, in contrast to DNA detection methods which generally do not allow this distinction to be made (Bentsink et al. 2002; Scuderi et al. 2010). Several authors, however, have described NASBA assays which display some DNA amplification activity (Voisset et al. 2000; Rodriguez-Lazaro et al. 2004), so if complete specificity for RNA is required it may be necessary to also use a nucleic acid extraction method that is selective for RNA. NASBA requires denaturation of the template to allow primer annealing prior to the addition of non-thermostable enzymes, making reaction set-up a two-stage process.

In the context of plant pathogen detection, NASBA has primarily been applied to the detection of RNA viruses (for example, Klerks et al. 2001; Vašková et al. 2004; Olmos et al. 2007), but also some bacterial pathogens (Bentsink et al. 2002; van Beckhoven et al. 2002; Van der Wolf et al. 2004; Scuderi et al. 2010), where the ability to discriminate between viable and non-viable cells can be an advantage for some applications. NASBA is considered

to be a highly sensitive detection method with relatively short reaction times (typically 90 minutes); however, fluorescent detection in conjunction with NASBA has generally utilised instrumentation which is not suitable for non-laboratory use. The need for a two-step protocol also makes NASBA less suitable for on-site use, as even a single additional manipulation can make a method too complex for use in some scenarios, and also greatly increases the likelihood of contamination.

1.5.3 Other isothermal amplification methods

Two further isothermal amplification methods which have been applied to the detection of plant pathogens are RCA and ICAN. In its simplest format, RCA is used to amplify circular nucleic acids utilising the strand displacement activity of Phi29 DNA polymerase. RCA followed by restriction fragment length polymorphism (RFLP) analysis has been used for diagnosis of geminiviruses (Haible et al. 2006) which have small single-stranded circular DNA genomes. More complex methods based on RCA make use of circular probes (Murakami et al. 2008) or circularisable padlock probes (Banér et al. 1998) to provide templates for amplification.

ICAN uses 5′-DNA-RNA-3′ chimeric primers with a thermophilic RNase H which introduces a nick at the junction between the DNA and RNA portions of the primers, and a DNA polymerase with strand displacing activity which continues extension from the nick site (Mukai et al. 2007; Uemori et al. 2007). Urasaki et al. (2008) demonstrated the use of ICAN with a chimeric RNA-DNA cycling probe for end-point detection of *Candidatus* Liberibacter asiaticus, with a reaction time of 1 hour.

1.5.4 Loop-mediated isothermal amplification

The isothermal amplification methods discussed so far each have various advantages. However, in the context of developing methods for on-site use, factors such as reaction time (>1 hour in the case of HDA, NASBA, and ICAN) and complexity of assay design (RPA, RCA for non-circular targets) are potential disadvantages. An alternative isothermal amplification approach is to design primers such that the amplification products contain single stranded primer binding sites. LAMP is the most commonly used method to take this approach, using three pairs of primers (internal, external and loop primers), as shown in Figure 1.2, to generate an amplification product which contains single-stranded loop regions to which primers can bind without template denaturation (Notomi et al. 2000; Nagamine et al. 2002) at a reaction temperature of around 65°C. The internal primers introduce self-complementarity into the amplification product, causing loops to form, while extension of the external primers causes displacement of the extension products of the internal primers. The products of LAMP reactions consist of alternately oriented repeats of the target sequence, resulting in a characteristic ladder-like appearance when visualised by gel electrophoresis (Notomi et al. 2000). The addition of loop primers was described by Nagamine et al. (2002) to accelerate amplification by priming at the loop regions that are of the incorrect orientation for the internal primers to bind (Figure 1.2). Loop primers increase sensitivity and reduce reaction times, and are required for acceptable performance of some assays. However, to accommodate loop primers requires a longer region of suitable sequence, such that design of two loop primers may not be possible, and many assays have been reported in the literature which

achieve acceptable performance without loop primers, or with only one loop primer. Gandelman et al. (2011) recently described a further modification of the LAMP reaction incorporating one or more ‘stem’ primers, which bind to the double stranded central portion of each repeat of the amplified region, to further enhance assay performance and increase primer design options. Because LAMP uses at least six primer binding regions, it is possible to design assays with high specificity by positioning each primer at the site of mismatches between the target and non-target species. LAMP assays have been reported with sensitivity approaching that of comparable real-time PCR assays (Tomlinson et al. 2007), and typically exceeding that of conventional PCR (Fukuta et al. 2003b; Zhang et al. 2011).

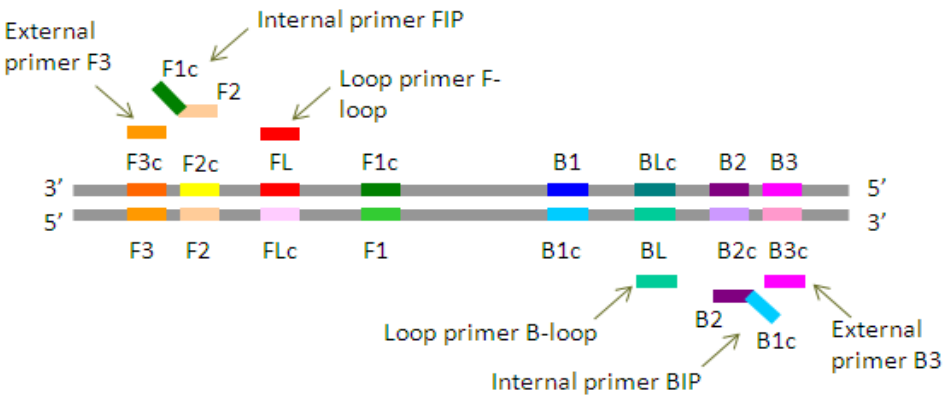

Figure 1.2 Configuration of primers for loop-mediated isothermal amplification (LAMP). Six primer binding sites are required for LAMP (F1, F2 and F3 plus B1, B2 and B3), with the internal primers FIP and BIP each targeting two sequences (F1 and F2, and B1 and B2, respectively). Loop primers (F-loop and B-loop) can be added to accelerate amplification.

LAMP does not require initial template denaturation (Nagamine et al. 2001), but several groups have reported that sensitivity is increased for some assays by the inclusion of a brief incubation at 95°C (Suzuki et al. 2010), in common with other isothermal methods (NASBA, HDA) which either require or benefit from initial denaturation. The enzymes typically used for LAMP must be added after the denaturation step; however, as discussed previously, this additional manipulation poses a contamination risk and is best avoided for on-site testing. Initial descriptions of LAMP used *Bst* polymerase with typical reaction times in the range 60 to 90 minutes. More recently developed strand displacing DNA polymerases display faster reaction kinetics, such that LAMP reaction times can be reduced to <30 minutes. LAMP, in common with other isothermal DNA amplification methods, can be modified for detection of RNA targets by the addition of reverse transcriptase to the reaction. In RT-LAMP, reverse transcription and amplification of cDNA proceed concurrently at a single temperature of around 65°C. Significantly for on-site testing, LAMP has been reported to be tolerant of some substances which are inhibitory to PCR (Kaneko et al. 2007; Tani et al. 2007c), potentially allowing LAMP to be used in conjunction with simplified nucleic acid extraction methods.

In summary, numerous approaches to the isothermal amplification of nucleic acid have been developed, with a small number becoming fairly well established. In the specific context of on-site testing for plant pathogens, some assay characteristics are particularly desirable, including speed of amplification, simplicity of workflow (i.e. requiring few manipulations) and tolerance of inhibitors, all of which are exhibited by LAMP.

1.6 Detection of LAMP products

A nucleic acid-based test for a particular target comprises not only the mechanism for nucleic acid amplification, but also a means of determining whether amplification has occurred. Some detection methods are broadly applicable and can be used to detect the products of diverse amplification methods including LAMP. The extremely high efficiency of LAMP, however, results in the generation of sufficiently large amounts of amplification product to allow the use of relatively insensitive detection methods which cannot be used with less efficient amplification chemistries. A common and broadly applicable method for detection of amplification products is gel electrophoresis; however, this is too cumbersome and time consuming for use outside the laboratory. Another approach is to monitor amplification in real-time using fluorescent dyes or probes, as discussed previously in the context of real-time PCR. The requirement for rapid heating and cooling as well as fluorescence monitoring adds considerable complexity to instruments for real-time PCR, but real-time fluorescence monitoring of isothermal methods can be achieved using simpler and less costly instruments, including the OptiGene Genie II and TwistDx Twista. The most common method for real-time fluorescence monitoring of LAMP reactions uses intercalating dyes such as SYBR Green (Maeda et al. 2005; Ohtsuka et al. 2005), but the use of labelled probes and primers has also been described (Kouguchi et al. 2010; Zerilli et al. 2010; Chou et al. 2011). Fluorescence detection using intercalating dyes has the advantage of allowing further analysis in terms of the temperature at which amplification products melt or anneal. LAMP products contain structures of differing lengths containing catenated repeats of the target sequence which

melt / anneal at a specific temperature determined by the length and G/C content of the target. After amplification, reactions can be subjected to a gradual melting or annealing step with fluorescence monitoring in order to discriminate specific amplification products from non-specific artefacts.

Another consequence of the high amplification efficiency of LAMP is that the generation of magnesium pyrophosphate (a by product of DNA polymerisation) causes a measurable increase in turbidity as the reaction proceeds, allowing amplification to be monitored in real-time using relatively simple instruments for continuous turbidity measurement (Mori et al. 2004). As an alternative to the measurement of turbidity, pyrophosphate generation can be monitored indirectly in real time via a bioluminescent reaction using thermostable firefly luciferase in a technology referred to as BART (bioluminescent assay in real-time) (Gandelman et al. 2010).

In fact, magnesium pyrophosphate is generated in such high quantities in LAMP reactions that the precipitate formed is visible to the naked eye (Mori et al. 2001), allowing positive and negative reactions to be identified simply by inspection of the reaction tube. Since no post-amplification manipulation is required, this method presents a lower risk of carry-over contamination than methods which require reaction tubes to be opened (such as gel electrophoresis). However, visual detection of the white precipitate is somewhat subjective, and may not give conclusive results for all assays (Wastling et al. 2010). A number of colour change reactions can be used for end-point detection, including the addition of intercalating dyes such as SYBR Green and PicoGreen at sufficiently high concentrations to produce a visible colour change (Iwamoto et al. 2003; Dukes et al. 2006; Tomlinson et al. 2007),

or the addition of fluorescent probes and cationic polymers (Mori et al. 2006). However, at the concentrations required these reagents are inhibitory to LAMP so must be added at the end of the reaction, with a high risk of carry-over contamination. In order to allow SYBR Green to be used in a closed-tube format, Tao et al. (2011) used microcrystalline wax to encapsulate the dye, with a heating step after amplification to melt the wax and release the dye into the reaction. Alternative colour change reactions have been described using reagents which do not inhibit amplification, allowing them to be used in a closed-tube format. These include calcein plus $MnCl_2$, which causes a colour change from orange to green (Tomita et al. 2008), and hydroxy naphthol blue (HNB), which results in a colour change from violet to blue (Goto et al. 2009). The colour change with calcein and $MnCl_2$ has been reported to be more difficult to interpret than other methods (Wastling et al. 2010), and in any case is best viewed under ultra violet illumination. The colour change with HNB has been reported to be easily interpretable by end-users (Wastling et al. 2010), but the change can be subtle and clarity of the results may be somewhat assay-dependent (Tomlinson et al. 2010a).

An alternative method for detection of amplification products is the incorporation of ligands during amplification which can be detected in an LFD immunoassay at the end of the reaction. The ligands can be incorporated using labelled nucleotides or primers for amplification, or probes added at the end of the reaction. Variations of this detection method have been described for PCR (Srisala et al. 2008), LAMP (Kiatpathomchai et al. 2008; James et al. 2009), HDA (Goldmeyer et al. 2008) and RPA (Rohrman and Richards-Kortum 2012). Amplicon detection using this method typically requires reaction tubes

to be opened to allow the amplified product to be applied to the LFD. Devices are available which incorporate LFD detection in a closed cassette (Goldmeyer et al. 2008), but the per-device cost is higher than using LFDs in either a simple dipstick format or conventional open housing.

1.7 LAMP methods – variations

LAMP has been used in combination with a number of other technologies, with the expectation of maximising the advantages conferred by each method. For example, LAMP has been combined with padlock probes and RCA (Marciniak et al. 2008), with the aim of increasing the speed of detection while retaining the advantages of padlock probe-based methods (high specificity and options for subsequent detection of generic probe elements), and with NASBA (Fukuda et al. 2008) to maximise sensitivity. In both cases, however, the overall reaction times were over 3 hours. Various laboratory-based technologies have been used for interrogation of LAMP products, including dot-blot hybridization (Teng et al. 2007), macroarrays (Inácio et al. 2008) and pyrosequencing (Liang et al. 2012). Finally, LAMP has been combined with ELISA, to increase throughput for routine testing (Ravan and Yazdanparast 2012), and with solid-phase proximity ligation for detection of a protein target (Jiang et al. 2012). In most cases, however, combination with other technologies undermines the primary advantages of LAMP for on-site testing, which are speed and simplicity of workflow.

In the context of on-site testing, modifications which address possible shortcomings of the basic LAMP method are potentially more useful. Reference has been made to the susceptibility of LAMP to carry-over

contamination, particularly when using non-homogeneous detection methods. To attempt to address this problem, He and Xu (2011) described the use of dUTP in LAMP reactions such that uracil-N-glycosylase (UNG) can be used to selectively degrade any amplification product that is inadvertently carried over to subsequent reactions. Despite relatively inefficient incorporation of dUTP by *Bst* polymerase, these authors reported the successful use of this method to prevent carry-over contamination in LAMP reactions.

As discussed in the context of using NASBA for amplification of RNA, another potential shortcoming of LAMP and other DNA amplification methods is the inability to discriminate between viable and dead cells. The DNA intercalating dyes propidium monoazide (PMA) and ethidium monoazide (EMA) penetrate dead cells where they bind to DNA, preventing subsequent amplification; conversely, the dye does not enter live cells, and the DNA remains amplifiable. Use of PMA or EMA treatment prior to LAMP for selective detection of viable cells of *Salmonella* has been reported (Lu et al. 2009; Chen et al. 2011). PMA / EMA treatment is relatively rapid but requires accurate exposure of samples to light to allow cross-linkage of DNA in dead cells and photolysis of free molecules of dye, however, and this might not be easily achieved in field conditions.

1.8 Quantitative detection of plant pathogens

Quantitative detection is not necessary for some applications of on-site testing. For example, for detection of quarantine plant pathogens at inspection, it is generally only necessary to establish whether the target organism is present or absent. For other applications, however, and particularly for

detection of non-quarantine pathogens, quantitative information about pathogen levels may be more useful. Examples of such applications include testing for pathogens where information about inoculum levels influences decisions regarding crop management or the storage or processing of commodities such as fruits and vegetables. The degree of accuracy required is dependent on the application. Research into aspects of plant pathogen biology such as disease transmission or pathogen distribution may benefit from highly accurate quantification, but testing in this context is likely to be performed in the laboratory. For on-site detection, however, methods generating semi-quantitative results may be more applicable and more easily attained, not least because methods for extremely accurate quantification are typically more complex than those which are qualitative or semi-quantitative (for example, categorisation of pathogen levels as high, medium or low).

Quantitative detection in the laboratory is most commonly achieved using real-time PCR, in which the Ct value (cycle at which fluorescence exceeds a defined threshold) is proportional to the input amount of DNA in the reaction. Several quantification methods are available for absolute or relative quantification of DNA using real-time PCR, most of which require the construction of a standard curve by testing standards of known concentrations in parallel with the samples to be quantified (Wong and Medrano 2005). As discussed previously, LAMP reactions can be monitored in real-time by measurement of fluorescence, bioluminescence or turbidity; moreover, the time to positivity (Tp) value of a LAMP reaction is proportional to the input quantity of DNA. However, the short reaction times of LAMP using new, faster strand displacing polymerases can potentially make quantification based

on Tp less accurate, as amplification of templates over a fairly wide range of concentrations can occur within a short period of time. Nevertheless, quantitative LAMP assays using real-time detection have been reported for various targets (Mori et al. 2004; Mekata et al. 2009; Gandelman et al. 2010).

An alternative approach to quantitative detection is the use of co-amplifying competitive templates (Diviacco et al. 1992). In this approach, a competitor template is added to the reaction which is amplified by the same primers as the target DNA. The competitor is designed such that its amplification product can be distinguished from the amplification product of the target at the end of the reaction (for example, on the basis of length determined by gel electrophoresis). The ratio of target to competitor is maintained throughout the reaction, such that the target concentration can be calculated from the known amount of competitor and the ratio of the two amplified products. An advantage of quantification methods based on competitive amplification is that the presence of inhibitors has less effect on the accuracy of quantification in comparison with methods based on real-time detection, because any inhibitory effects apply equally to amplification of the target and the inhibitor. Quantification using competitive PCR (Manome et al. 2008; Miyata et al. 2010) and LAMP (Tani et al. 2007c) has been demonstrated in the presence of inhibitory substances typically present in soil extracts.

1.9 Multiplex detection of plant pathogens

A further consideration is the ability of a method to simultaneously detect multiple targets. Laboratory-based methods can achieve highly parallel detection of large numbers of targets, for example, using microarrays (Boonham et al. 2007), or non-targeted detection of disease-causing agents using next generation sequencing (Adams et al. 2009). For on-site use, however, the number of targets that must be detected in a single test is likely to be small. The development of methods for diverse targets which share a common workflow is more important than multiplex detection *per se* for applications such as testing at inspection, where the end-user may need detection tools for many targets, but will typically deploy those tools one at a time. However, even when a detection method targets an individual pest or pathogen, it is generally desirable to incorporate a control assay into the test to allow proper interpretation of negative results, since false negatives can be caused by inhibition or inefficient nucleic acid extraction. In routine testing for plant pathogens it is common practice to incorporate into the test an assay for detection of host plant nucleic acid, in order to verify that nucleic acid extraction was successful and that the extract is free from inhibitors (Weller et al. 2000; Korimbocus et al. 2002). In the case of abiotic matrices, or where DNA yields from the host are low, artificially constructed controls can be added to the amplification reaction, to control for inhibition, or to the sample prior to extraction, to control for both inhibition and extraction efficiency (Klerks et al. 2004; Coyne et al. 2005; Hartman et al. 2005).

Multiple targets can be detected in parallel (in separate reactions), but multiplex detection of more than one target in a single reaction can have the

advantages of reducing cost and increasing capacity (Martin et al. 2000). Unlike PCR products, LAMP products are not readily differentiated by gel electrophoresis without additional processing steps, such as treatment with restriction enzymes (Iseki et al. 2007). Multiplexing can be achieved by detection of differently-labelled fluorescent probes if suitable real-time fluorescence instrumentation is available. Potential approaches for on-site resolution of mixed LAMP products include analysis of amplicon melting temperatures using an instrument such as the Genie II and detection of differently labelled amplicons using LFDs.

1.10 Examples of LAMP for detection of plant pathogens and pests

LAMP assays have been developed for the detection of a variety of plant pathogens and pests (Tomlinson and Boonham 2008), including viruses (Fukuta et al. 2003a, b; Fukuta et al. 2004; Nie 2005; Varga et al. 2006), viroids (Boubourakas et al. 2009), fungi (Tomlinson et al. 2007; Niessen and Vogel 2010; Huang et al. 2011), bacteria (Kubota et al. 2008; Li et al. 2009; Harper et al. 2010; Moradi et al. 2012), phytoplasmas (Tomlinson et al. 2010a; Bekele et al. 2011; Hodgetts et al. 2011; Obura et al. 2011; Yankey et al. 2011) and liberibacter (Okuda et al. 2005; Li et al. 2007), as well as nematodes (Kikuchi et al. 2009; Niu et al. 2011) and insect pests (Huang et al. 2009; Arif et al. 2012; Hsieh et al. 2012). In most cases the rationale for using LAMP was the lower instrumentation costs compared to PCR or real-time PCR and the shorter reaction times of LAMP. The most commonly used detection methods in these reports are gel electrophoresis and end point observation of turbidity or colour changes. Some of the reports of LAMP for plant pathogen detection

have addressed the requirements for on-site testing more directly, in terms of the use of simplified extraction methods (for example, Fukuta et al. 2003a; Harper et al. 2010; Tomlinson et al. 2010a; Hadersdorfer et al. 2011; Li et al. 2011; Niu et al. 2011) and amplicon detection using LFDs (Kikuchi et al. 2009; Rigano et al. 2010). A small number of reports have gone on to elaborate on the potential value of LAMP-based methods in the context of disease management. For example, Temple and Johnson (2011) deployed a LAMP-based test for *Erwinia amylovora* and discussed the potential impact of testing using this method on forecasting and management of fire blight in pear and apple orchards.

The development of LAMP assays for plant pathogens is becoming more common in light of the potential advantages of LAMP in comparison with established methods. It will be necessary to consider how these tests can be deployed for maximum benefit. One element of this is ensuring that the methods are fit for purpose in terms of the basic characteristics of the test (for example, sensitivity and specificity, and whether results are qualitative or quantitative) and factors affecting the likelihood of adoption by the intended end-users, such as the speed to result, complexity of the workflow and the accessibility of equipment and reagents. In the context of testing for quarantine pests and pathogens, another important factor is the need to validate methods to acceptable standards (Martin et al. 2000; López et al. 2003; Miller et al. 2009). It will be necessary to take a strategic approach to how on-site tests with particular performance characteristics are deployed in the pursuit of specific objectives in the control and management of pests and diseases (López et al. 2009).

1.11 Validation and deployment of methods for on-site detection of plant pathogens

How a test is deployed in order to achieve specific objectives, and how the results of the test are interpreted, should be informed by the performance characteristics of the test (Olmos et al. 2008; López et al. 2009), such that the performance of a new test should be evaluated before it is used for routine testing. A further driver for this is the need for methods used for statutory testing by NPPOs to be validated as recommended by the appropriate regional plant protection organisation (in Europe, the European and Mediterranean Plant Protection Organisation (EPPO)). The EPPO protocol for laboratories preparing for accreditation (EPPO 2010) considers validation to comprise the description of analytical sensitivity and specificity, as well as repeatability and reproducibility. Analytical sensitivity is generally described in terms of a lowest detectable number of cells or colony forming units (CFU), amount of nucleic acid or dilution factor (in the case of pathogens which are not readily isolated from their hosts). Analytical specificity can be more difficult to define, and can include exclusion of non-target species known to be closely related to the target, as well as organisms which cause similar symptoms or are commonly found in the same host or matrix. Non-target species which are morphologically similar or cause similar symptoms may be unrelated (or only distantly related) to the target, such that the likelihood of cross reactivity can be adequately assessed *in silico*. Analytical specificity should be revisited periodically due to the emergence and description of new taxa; for some targets this also applies to inclusivity for detection of all relevant strains or subspecies.

Approaches to validation often also involve a comparison of the new method with a previously defined standard method. In a comparative test, diagnostic sensitivity and specificity describe how many of the positive and negative samples were correctly identified as such by the new test. However, these measures do not reflect the proportion of positive results obtained that were correct (referred to as the positive predictive value) or the proportion of negative results that were correct (the negative predictive value), which are more intuitive measures from the diagnostician's perspective (Loong 2003; López et al. 2009). Predictive values are significantly influenced by the prevalence of the pathogen in the samples tested, but this is typically heavily manipulated by the selection of samples for comparative testing for validation. For example, the EPPO guidelines recommend that comparative testing should be carried out using a set of samples of which 33% to 50% are infected with the target pathogen (EPPO 2010). At 50% infection, the effect of prevalence on predictive values is minimal, but the actual rate of infection might be very different when real samples are tested. As an illustration, if a test with diagnostic sensitivity of 90% and diagnostic specificity of 90% is used to test samples from a population where prevalence of the pathogen is 10%, approximately half of all positive results will be false positives; conversely, if prevalence of the pathogen is 90%, approximately half of all negative results will be false negatives (see Figure 1.3).

A

New method	Standard method: +	Standard method: -	total
+	45	5	50
-	5	45	50
total	50	50	

B

New method	Standard method: +	Standard method: -	total
+	9	9	18
-	1	81	82
total	10	90	

C

New method	Standard method: +	Standard method: -	total
+	81	1	82
-	9	9	18
total	90	10	

Figure 1.3 Effect of prevalence on the interpretation of results for a new detection method, based on performance characteristics evaluated by comparative testing. The results of the standard method are assumed to accurately reflect disease status. In each case, diagnostic sensitivity (the proportion of positive samples which test positive by the new method) = 90% and diagnostic specificity (proportion of negative samples which test negative by the new method) = 90%. If 50% of samples are infected, the positive predictive value (proportion of samples testing positive using the new method that are truly positive) = 90% and the negative predictive value (proportion of samples testing negative using the new method that are truly negative) = 90% (**A**). If only 10% of samples are infected, however, the positive predictive value is only 50% (**B**) i.e. half of the positive results recorded are false positives; conversely if 90% of the samples are infected, the negative predictive value is 50% (**C**).

The ‘prevalence’ of the pathogen in the samples being subjected to testing will only reflect the actual prevalence of the pathogen in the environment if samples are randomly selected. In most on-site testing situations, however, the samples for testing will be selected on the basis of observation of symptoms, such that the prevalence of the pathogen in the samples reaching the test will be higher than in the host population in general (but could still be low if the symptoms are non-specific). The performance characteristics of visual assessment as a primary screen are rarely taken into account, and in any case may be highly variable between operators. Beyond this, if the on-site test is in turn used as a screen to select samples for subsequent laboratory testing, the performance characteristics of the on-site test will have a knock-on effect on how the results of the laboratory test should be interpreted. For example, where a primary screen is highly specific, such that most of the samples submitted to the next level of testing are positive, negative results have an increased chance of being false negatives, and additional confirmatory testing may be desirable. In order to fully evaluate the impact of methods for the on-site detection of plant pathogens it will therefore be necessary to take into account all elements of the diagnostic process, from visual inspection and selection of samples in the field to any subsequent confirmatory testing in the laboratory.

1.12 The aims and objectives of this study

The aim of this project was to investigate aspects of on-site detection for plant pathogens by developing methods suitable for deployment in a range of scenarios.

Specific goals were:

1. to investigate the integration of rapid DNA extraction using *Phytophthora* spp. lateral flow devices (LFDs) with prescreening by LFD immunoassay and routine laboratory testing for *P. ramorum* (Chapter 2);

2. to investigate the use of rapid DNA extraction using LFDs in conjunction with loop-mediated isothermal amplification (LAMP) for *P. ramorum* and *P. kernoviae* followed by LFD detection of incorporated ligands (Chapter 3);

3. to develop LAMP assays for detection of the following non-quarantine and quarantine plant pathogens, with an emphasis on developing methods appropriate to the relevant applications:

    a. the non-quarantine fungal pathogen *Botrytis cinerea*, with a specific focus on real-time fluorescence detection and detection of pre-symptomatic infection (Chapter 4);

b. *Guignardia citricarpa*, the EPPO-listed causal agent of citrus black spot disease, with a specific focus on development of a workflow suitable for confirmation of symptoms at import inspection (Chapter 5);
c. *Cassava brown streak virus* and *Ugandan cassava brown streak virus*, causal agents of cassava brown streak disease in East Africa, with a specific focus on development of a non-instrumented method for detection of both viruses, and comparison with existing methods (Chapter 6);

4. to investigate the use of a competitive end-point quantitative LAMP method, using *B. cinerea* as a model target (Chapter 7).

CHAPTER 2

Published in the Journal of Microbiological Methods, 2010

J.A. Tomlinson, M. Dickinson, E. Hobden, S. Robinson, P.M. Giltrap and N. Boonham. (2010) A five-minute extraction method for expedited detection of *Phytophthora ramorum* following prescreening using *Phytophthora* spp. lateral flow devices. *Journal of Microbiological Methods*, 81, 116-120

Author contribution:

Project supervised by M. Dickinson and N. Boonham.

Routine diagnostic testing performed by E. Hobden, S. Robinson, P.M. Giltrap (Fera Molecular Testing Unit & Fera Mycology sub-team).

All other data collected and analysed and manuscript prepared by J.A. Tomlinson.

A five-minute DNA extraction method for expedited detection of *Phytophthora ramorum* following prescreening using *Phytophthora* spp. lateral flow devices

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ABSTRACT

In a direct comparison with established methods for *Phytophthora ramorum* detection (isolation followed by morphological identification, or conventional DNA extraction followed by TaqMan real-time PCR) a rapid, simplified detection method in which membranes of lateral flow devices (LFDs) are added directly to TaqMan real-time PCR reactions was used to test 202 plant samples collected by plant health inspectors in the field. *P. ramorum* prevalence within the 202 samples was approximately 40% according to routine testing by isolation or TaqMan real-time PCR. The diagnostic sensitivity and specificity of the rapid detection method were 96.3% and 91.2%, respectively. This method can be used in conjunction with *Phytophthora* spp. lateral flow devices to reduce the number of samples

requiring testing using more laborious conventional methods. The effect of combining prescreening for *Phytophthora* spp. with *P. ramorum*-specific tests is discussed in terms of the positive and negative predictive values of species-specific detection when testing samples collected in different inspection scenarios.

Keywords: diagnostic sensitivity; diagnostic specificity; DNA isolation; real-time PCR; sudden oak death.

INTRODUCTION

Phytophthora ramorum is the causal agent of die back and leaf blight of a wide range of ornamental plants (principally rhododendron) in the UK and throughout Europe (Werres et al. 2001) and is also the cause of extensive oak mortality ('sudden oak death') on the west coast of North America (Rizzo et al. 2002). EU-wide emergency measures were implemented in 2002 (European Union 2002), and in the UK there is an ongoing programme of surveillance for the presence of this pathogen by Defra's Plant Health and Seeds Inspectorate (PHSI), who have the authority to enforce eradication and containment measures including the destruction of infested material. A number of methods have been developed for the nucleic acid-based detection of *P. ramorum*, including several based on real-time PCR (Hayden et al. 2004; Tomlinson et al. 2005; Hayden et al. 2006; Hughes et al. 2006b; Schena et al. 2006; Tooley et al. 2006; Bilodeau et al. 2007) These methods have been found to have high specificity and sensitivity, detecting less than 12 fg *P. ramorum* DNA (Hayden et al. 2004), and can be used for testing both cultured pathogen and infected plant material. The majority of assays reported to date have been used in conjunction with DNA extraction methods based on spin columns or processing of magnetic beads (EPPO 2006; Hughes et al. 2006b; Tooley et al. 2006; Bilodeau et al. 2007; Kox et al. 2007) or using organic solvents such as phenol and chloroform (Hayden et al. 2004; EPPO 2006; Schena et al. 2006). These methods generally result in high quality DNA extracts, but they are also time consuming even when automated for high-thoughput use.

In England and Wales, samples of plant material taken by plant health inspectors are sent to the Food and Environment Research Agency (Fera) for

P. ramorum testing. The diagnostic method used in the laboratory depends primarily on the host plant: the majority of rhododendron samples are tested directly by TaqMan real-time PCR (Hughes et al. 2006b), while other hosts are tested by plating of plant material on semi-selective media followed by morphological examination. In accordance with the EPPO diagnostic protocol for *P. ramorum* (EPPO 2006), positive identification of the pathogen is possible on the basis of an unambiguous result for either real-time PCR or morphological examination. In practice, only samples of the most common host in the UK (rhododendron) are tested by real-time PCR, and any ambiguous real-time PCR results are confirmed by isolation and morphological examination. Further to this, partial sequencing of the ITS region of the rRNA gene is carried out to confirm the identity of the pathogen in samples from new outbreak sites and in previously unrecorded hosts.

Phytophthora spp. lateral flow devices (LFDs) are used by some plant health inspectors for screening samples in the field (Lane et al. 2007). The use of these devices has been found to be a suitable pre-screening method (Kox et al. 2007; Lane et al. 2007) due to the high diagnostic sensitivity of this method compared to methods which identify *P. ramorum* at the species level (cultural and/or PCR-based methods). Pre-screening reduces the number of samples sent to the laboratory for testing, resulting in a considerable cost saving, but several thousand samples are still sent to the laboratory every year. Sending samples to the laboratory for testing has a number of disadvantages including the movement of potentially infectious material away from outbreak sites; a requirement for stringently observed quarantine procedures at the testing

laboratory; and the possibility of discrepant results due to uneven distribution of the pathogen or degradation of samples in transit.

Fera have developed a method for extraction of nucleic acid from plant material using LFDs (Danks and Boonham 2007). A section of the LFD nitrocellulose membrane can be added directly to a DNA amplification reaction, such as real-time PCR, without any additional processing. LFDs run at inspection sites could be sent to the laboratory for testing by TaqMan real-time PCR. This approach would obviate the need to send plant material to the laboratory and has the advantage of expediting real-time PCR testing, since conventional DNA extraction is not required. In order to evaluate the potential utility of this approach, 202 samples sent to the laboratory at Fera for routine testing for *P. ramorum* were also tested by DNA extraction using the LFD method followed by real-time PCR. The results were compared to those obtained by routine testing using established methods.

MATERIALS AND METHODS

Samples

Samples of plant material with suspected symptoms of *P. ramorum* were collected by the PHSI as part of ongoing surveillance for *P. ramorum*. 198 out of 202 samples were leaf material, as recorded by the diagnostician who received the sample (the remaining samples were recorded as stem/shoot, leaf/twig/branch, or leaf litter). Samples were dispatched from the field in sealed plastic bags containing a small piece of damp tissue. On receipt in the laboratory, the material was examined for the presence of typical symptoms, and sub-samples were taken from the leading edge of any identified lesions.

Sub-sampled material was washed briefly in distilled water to remove any debris from the surface. Samples were predominantly rhododendron (141 samples) but also included *Pieris* (14 samples), *Viburnum* (seven samples), *Magnolia* (seven samples) and *Camellia* (six samples).

Routine laboratory testing

Samples were tested either by plating on semi-selective media followed by morphological assessment of any growth, or by DNA extraction directly from the plant material using a magnetic bead-based extraction method followed by TaqMan real-time PCR. This is in accordance with the EPPO diagnostic protocol (EPPO 2006), in which a sample can be identified as positive on the basis of an unambiguous result obtained by either real-time PCR or morphological examination. The majority of rhododendron samples (113 out of 141 samples) were initially tested directly by TaqMan. The remaining rhododendron samples were tested by culturing if the sample originated from a previously unrecorded outbreak site or if there was considered to be insufficient material to allow subsequent culturing if the TaqMan result was ambiguous. All non-rhododendron hosts were tested by culturing only. Following assessment of symptoms, excised pieces of tissue were tested immediately by either conventional DNA extraction followed by TaqMan real-time PCR or culturing on semi-selective media. Duplicate samples were stored at 4ºC, prior to testing by LFD followed by TaqMan real-time PCR.

For detection by culturing, pieces of tissue were plated out on P_5ARP[H] semi-selective media (as described by Jeffers and Martin, 1986).

The plates were examined microscopically after 6 days for the presence of *P. ramorum* growth (Werres et al. 2001).

Alternatively, DNA was extracted from the material using a KingFisher ML platform (Thermo Scientific, Waltham, MA). Briefly, samples (typically 200 - 500 mg) were homogenized in 10 volumes of Buffer C1 from the NucleoSpin Plant kit (Machery Nagel, Düren, Germany), incubated at 65°C for 30 minutes and centrifuged for 2 minutes at 6000 x g. DNA was extracted from the clarified lysates by adding 1 ml PB Binding Buffer (Qiagen, Hilden, Germany) and 75 µl Magnesil paramagnetic particles (PMPs) (Promega, Madison, WI) and processing the samples using a KingFisher ML to wash the PMPs three times in 70% ethanol and elute the DNA in 200 µl molecular grade water.

DNA extracts were tested by TaqMan real-time PCR for *P. ramorum* and plant cytochrome oxidase (COX) using primers and probes described by Hughes et al. (2006b), as shown in Table 2.1. Real-time PCR was carried out on an ABI 7900HT (Applied Biosystems, Foster City, CA) using TaqMan Core Reagents (Applied Biosystems) as described by Hughes et al. (2006b), except that the *P. ramorum* and COX reactions were carried out in separate wells. Samples for which the COX TaqMan Ct value >28 or the *P. ramorum* Ct value >36 were retested by culturing, the result of which was taken as the final result.

Table 2.1. Primers and probes used for TaqMan real-time PCR[1].

Primer / probe	Sequence (5′-3′)	Reporter (5′)	Quencher (3′)	Final concentration (nM)
Pram-114F	TCATGGCGAGCGCTTGA			300
Pram-114Fc	TCATGGCGAGCGCTGGA			300
Pram-190R	AGTATATTCAGTATTTAGGAATGGGTTTAAAAAGT			300
Pram probe	TTCGGGTCTGAGCTAGTAG	FAM[2]	BHQ1[3]	100
COX F	CGTCGCATTCCAGATTATCCA			300
COX RW	CAACTACGGATATATAAGRRCCRRAACTG			300
COX probe	AGGGCATTCCATCCAGCGTAAGCA	VIC[4]	TAMRA[5]	100

[1]Routine laboratory testing for *P. ramorum* was carried out using primers Pram-114Fc and Pram-190R, as described by Hughes at al. (2006b). Lateral flow devices were tested for *P. ramorum* using Pram-114F and Pram-190R, as described by Tomlinson et al. (2005).

[2]6-carboxyfluorescein

[3]Black Hole Quencher 1 (Biosearch Technologies, Novato, CA)

[4]Applied Biosystems, Foster City, CA

[5]tetra-methylcarboxyrhodamine

LFD DNA extraction and TaqMan real-time PCR

Phytophthora spp. LFDs were obtained from Forsite Diagnostics Ltd (York, UK). Samples (typically 200 – 500 mg) were placed in bottles containing 5 ml LFD Buffer C and five ball bearings (5 mm diameter) and shaken or vortexed for 2 to 3 minutes. Approximately 60 µl of Buffer C from the bottle was run on an LFD and the result recorded after five minutes. Positive results are indicated by the formation of two lines on the device; negative results are indicated by a single control line. Devices were left at room temperature for several hours or overnight before testing by TaqMan real-time PCR. For real-time PCR testing, the devices were dismantled and sections (approximately 1.5 mm by 2 mm) were cut from the membrane and added directly to TaqMan real-time PCR reactions. Sections were generally taken from the centre of the membrane, although it is not necessary to sample from any particular region of the membrane (Danks and Boonham 2007). Membranes were tested for *P. ramorum* and plant DNA (cytochrome oxidase) using the *P. ramorum* primers and probe used by Tomlinson et al. (2005), shown in Table 2.1, and the COX primers and probe described above. A base substitution was introduced into the forward primer Pram-114Fc used for routine testing and described by Hughes et al. (2006b) in order to increase discrimination between *P. ramorum* and the closely related pathogen *P. lateralis* when testing highly concentrated DNA extracted from cultures. As a result of this mismatch, the Ct values obtained using this primer are higher than recorded for the perfect match primer Pram-114F. The LFD extraction method results in the addition of smaller amounts of DNA to the real-time

PCR reaction, so the perfect match primer Pram-114F was used when testing LFD membranes (Tomlinson et al. 2005).

Real-time PCR reactions were set up in 96-well plates using TaqMan Core Reagents (Applied Biosystems) consisting of 1 x Buffer A and 0.025 U μl^{-1} AmpliTaq Gold, plus 0.2 mM each dNTP, 5.5 mM $MgCl_2$, 300 nM forward primer, 300 nM reverse primer, and 100 nM probe. The final volume of each reaction was 25 µl, and all reactions were carried out in duplicate. DNA extracted using conventional procedures was used as a positive control, and negative controls containing nuclease-free water instead of DNA were included in every run. Real-time PCR was carried out on an ABI Prism 7900HT (Applied Biosystems) using cycling conditions of 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, and results were analyzed using default threshold settings.

RESULTS

Results of routine laboratory testing

Results were obtained for all 202 samples tested using either TaqMan real-time PCR or morphological examination. Twenty four samples gave real-time PCR results which were considered to be ambiguous and were subsequently retested by isolation. Out of 202 samples, 81 were identified as positive for *P. ramorum* using conventional testing methods (*P. ramorum* prevalence 40.1%).

LFD DNA extraction method

Positive COX results (Ct value <40) were recorded for 186 out of 202 samples (Table 2.2). Of these, the Ct values for the majority of samples (182) were below 37. COX Ct values varied substantially between different hosts, and between samples from the same host, ranging from 25.19 to 39.13. Of the 16 samples which gave negative COX results, seven were positive for *P. ramorum* (Ct value <40). A sample was not required to have a positive internal control result if the *P. ramorum* result was positive, since the purpose of the COX assay was to allow interpretation of negative *P. ramorum* results. In total, the LFD extraction method failed for nine samples (negative for both COX and *P. ramorum*). Since the whole samples were destructively tested, re-extraction was not possible if the initial LFD extraction failed; in the course of routine laboratory testing, however, surplus material is retained to allow retesting if necessary.

Table 2.2. Extraction of DNA from a broad range of plant species using the LFD extraction method. LFDs were tested by real-time PCR for plant cytochrome oxidase (COX).

Recorded host name	Total number of samples	Number of samples: COX Ct[1] <37	COX Ct[1] 37-40	*P. ramorum* positive only	Failed extraction
Abelia	1	1	0	0	0
Acacia malanoxylon	1	1	0	0	0
Arbutus	1	1	0	0	0
Arbutus unedo	3	3	0	0	0
Camellia	4	2	0	1	1
Camellia japonica	2	1	1	0	0
Choisya	1	1	0	0	0
Decaisnea fagesii	1	1	0	0	0
Drimys	1	0	0	0	1
Fagus	1	1	0	0	0
Gaultheria	1	1	0	0	0
Kalmia	1	1	0	0	0
Kalmia latifolia	2	2	0	0	0
'Laurel type'	1	1	0	0	0
Laurus nobilis	1	1	0	0	0
Lomatia	1	1	0	0	0
Machilus breviflora	1	1	0	0	0
Magnolia	1	1	0	0	0
Magnolia grandiflora	3	3	0	0	0
Magnolia stellata	2	2	0	0	0
Magnolia x loebneri	1	1	0	0	0
Osmanthus burkwoodii	1	1	0	0	0
Pieris japonica	4	4	0	0	0
Photinia fraseri (*Photinia* x *fraseri*)	1	1	0	0	0
Pieris	10	9	0	1	0
Prunus laurocerasus	3	3	0	0	0
Quercus cerris	1	1	0	0	0
Quercus ilex	1	1	0	0	0
Rhododendron	112	98	3	5	6
Rhododendron ponticum	27	26	0	0	1
Rhododendron yakushimanum hybrids	2	2	0	0	0
Umbellularia californica	1	1	0	0	0
'Unknown'	1	1	0	0	0
Viburnum	2	2	0	0	0
Viburnum davidii	1	1	0	0	0
Viburnum tinus	4	4	0	0	0

[1]Mean Ct value for duplicate reactions.

P. ramorum detection using the LFD extraction / TaqMan method

Table 2.3 shows a summary of the results for the 193 samples for which results were obtained by both routine testing and the rapid LFD extraction / TaqMan method (COX and/or *P. ramorum* Ct <40). Of these samples, three false negative and 10 false positive results were obtained using the rapid method, resulting in a diagnostic sensitivity of 96.3% and a diagnostic specificity of 91.2% in comparison with routine testing.

Table 2.3. Comparison of the LFD extraction / TaqMan method and routine laboratory methods (isolation on semi-selective media or conventional DNA extraction followed by TaqMan) for detection of *P. ramorum*. Table shows positive (+) and negative (-) results for 193 samples tested using both methods. Results were not obtained for nine out of 202 samples (4.5%). Diagnostic sensitivity (A/A+C) = 96.3%; diagnostic specificity (D/B+D) = 91.2%.

		Routine testing (isolation or real-time PCR)		
LFD extraction / TaqMan		+	-	Total
	+	77 (A)	10 (B)	87
	-	3 (C)	103 (D)	106
	Total	80	113	193

The use of a conservative Ct value cut-off has been reported on a number of occasions (EPPO 2006; Hayden et al. 2006; Hughes et al. 2006b; Kox et al. 2007) since detection of very low levels of pathogen (reflected by high Ct values) may reflect cross-contamination of samples or extracts. Using this approach, samples for which high Ct values are recorded are not classified as positive or negative, but rather are considered to require further testing to obtain an unambiguous result. The use of a cut-off above which results are considered to require confirmatory testing has been recommended when performing routine testing of field samples (EPPO 2006). However, the value of the recommended cut-off is essentially arbitrary. Figure 2.1 shows the effect of using different cut-off values on sensitivity, specificity, and the percentage of samples which would require further testing. On the basis of Figure 2.1, a cut-off of 39 could be used to increase specificity, since four of the 10 false positive results had a Ct value greater than 39, without greatly increasing the number of samples which would require further testing.

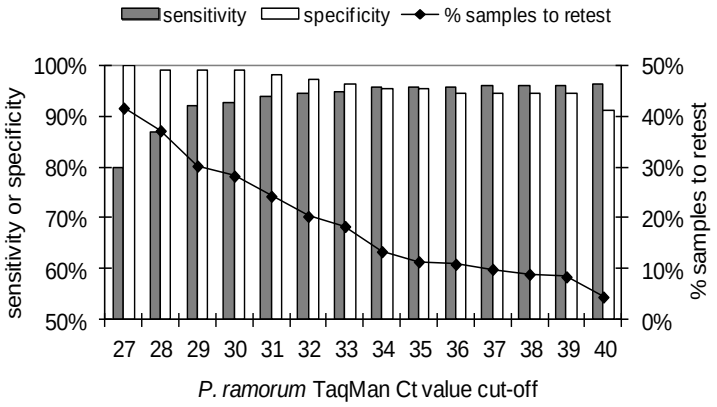

Figure 2.1. Effect of using different *P. ramorum* TaqMan Ct value cut-offs on diagnostic sensitivity and specificity of the LFD extraction / TaqMan method, and percentage of samples requiring retesting because no result was obtained (because both the *P. ramorum* and COX TaqMan results were negative, or because the *P. ramorum* Ct value exceeded the cut-off). 40 cycles indicates no cut-off.

Predictive values

As well as sensitivity and specificity, which reflect the likelihood that the correct result will be obtained for positive and negative samples, respectively, the performance of a detection method can also be evaluated in terms of predictive values (Kox et al. 2007; Vettraino et al. 2009). The positive and negative predictive values (PPV and NPV) reflect the likelihood that a positive or negative result reflects the true status of the sample (assuming that all results obtained using the gold standard method are correct). Without the use of a *P. ramorum* Ct value cut-off, the positive and negative predictive values are 88.5% and 97.2%, respectively. As shown in Figure 2.1, introducing a *P. ramorum* Ct value cut-off of 39 increases the specificity of the LFD extraction / TaqMan method by reducing the number of false positive results. Using this cut-off, the PPV and NPV for the LFD extraction / TaqMan method are 92.4% and 97.2%, respectively, for the group of samples tested in this experiment (where *P. ramorum* prevalence was approximately 40%).

Since predictive values are dependent on the prevalence of the disease in the population being tested, if disease prevalence is very low and the majority of samples tested are negative, the NPV will be high (due to few false negatives) and the PPV will be decreased (due to more false positives). Conversely, where disease prevalence is very high the PPV will be high (few false positives) but the NPV will be decreased (more false negatives). For prevalence values between 40% and 70% the positive and negative predictive values for the LFD extraction / TaqMan method both exceed 90% (Table 2.4).

Table 2.4. Effect of disease prevalence on positive and negative predictive values for the LFD/TaqMan detection method using a Ct cut-off of 39 cycles (sensitivity 96.1%, specificity 94.5%). Actual prevalence was approximately 40%.

Prevalence (%)	Positive predictive value (%)	Negative predictive value (%)
30	88.2	98.2
40	92.1	97.3
50	94.6	96.0
60	96.3	94.1
70	97.6	91.1
80	98.6	85.7

PPV = sensitivity x prevalence / ([sensitivity x prevalence] + [(1 – specificity) x (1 – prevalence)]) and NPV = specificity x (1 – prevalence)/([(1 – sensitivity) x prevalence] + [specificity x (1 – prevalence)]).

Prescreening by *Phytophthora* spp. LFD

Testing by *Phytophthora* spp. LFD could be used as a prescreen such that only samples that test positive by LFD are subjected to further testing. Of 52 samples which tested negative by *Phytophthora* spp. LFD, two were positive for *P. ramorum* by conventional testing methods. Using this method as a prescreen would therefore reduce the number of samples to be tested from 202 to 150 and result in two false negative results. Figure 2.2 and Table 2.5 show the outcome for all 202 samples if the *Phytophthora* spp. LFD is used as a prescreen followed by testing using the LFD extraction / TaqMan method with a *P. ramorum* Ct value cut-off of 39. Using this approach, only 14 out of 202 samples require conventional testing, and 10 incorrect results are recorded (four false negatives and six false positives), compared to performing conventional testing on all 202 samples (where all results would be assumed to be correct). However, a more conservative approach may be adopted for statutory testing, for example, requiring confirmatory testing of positive results by a second method. If all positive results obtained by the LFD extraction / TaqMan method were required to be confirmed by conventional methods, the number of samples for conventional testing would be increased to 93 out of 202 samples, and four false negatives and no false positives would be recorded.

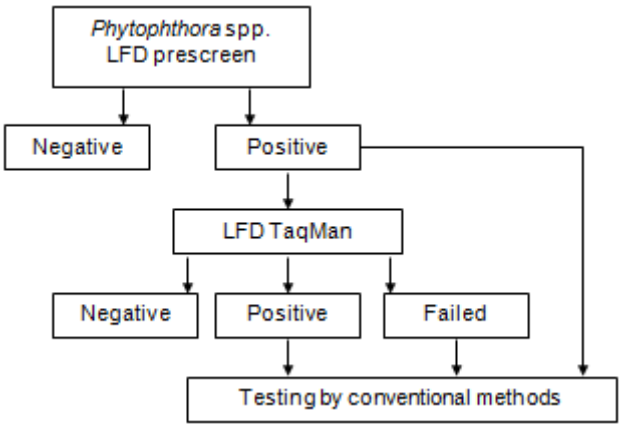

Figure 2.2. Summary of testing schemes comprising prescreening with *Phytophthora* spp. lateral flow devices; the LFD extraction / TaqMan method; and confirmation of positive results by conventional methods. Outcomes for the 202 samples tested are shown in Table 2.5.

Table 2.5. Summary of the number of false positive and false negative results, and the number of samples for requiring testing by conventional methods (DNA extraction by a conventional method followed by TaqMan real-time PCR or isolation and morphological examination), using the testing schemes outlined in Figure 2.2.

LFD prescreen	Testing method(s)	False positives	False negatives	No. of samples for conventional testing
No	Conventional methods	-	-	202
Yes	Conventional methods	-	2	150
Yes	LFD TaqMan[1]	6	4	14
Yes	LFD TaqMan[1] plus confirmation of positives by conventional methods	-	4	93

[1]Using *P. ramorum* Ct value cut-off of 39.

DISCUSSION

Conventional testing for *P. ramorum* can be laborious and time consuming, particularly for large numbers of samples. Isolation takes several days and success can vary with factors such as the time of year (Kox et al. 2007; Vettraino et al. 2009). Testing by real-time PCR can greatly reduce the time taken to obtain a result, but conventional DNA extraction prior to PCR can take several hours to complete, and automated extraction methods can be relatively costly. The LFD extraction / TaqMan method was found to have high diagnostic sensitivity (96.1%) and specificity (94.5%) when used with a *P. ramorum* Ct value cut-off of 39, allowing accurate results to be obtained without conventional testing for most samples. Direct testing of *Phytophthora* spp. LFDs which have been run in the field could minimize the amount of handling required in the laboratory since there would be no requirement for inspection of symptomatic plant material, plating out, or DNA extraction; pieces of LFD membrane could be added directly to real-time PCR reactions, taking less than 1 minute per sample. Furthermore, the likelihood of discrepancies between inspectors' observations in the field (i.e. results of *Phytophthora* spp. LFDs or observation of symptoms) and the results of laboratory testing is reduced since the actual device run by the inspector is subjected to real-time PCR testing in the laboratory.

A small number of false positive and false negative results were recorded using the LFD extraction / TaqMan method in comparison with conventional testing by isolation or real-time PCR. It is likely that at least some of these discrepant results reflect uneven distribution of the pathogen in the material that was split for testing by the routine and LFD extraction /

TaqMan methods. However, the specificity of isolation and real-time PCR are not known explicitly. In a comparison of *P. ramorum* detection methods including PCR-based methods, serological methods and isolation, Kox et al. (2007) defined positive samples on the basis of the results of either isolation or real-time PCR, an approach also used in the current EPPO diagnostic protocol (EPPO 2006). Both methods were therefore assigned a diagnostic specificity of 100% by definition (i.e. a sample did not have to test positive by both isolation and TaqMan in order to be designated as positive). The actual specificity of the individual methods used for routine testing is unknown, and this could underlie the three false negative results which were recorded using the LFD extraction / TaqMan method.

Table 2.2 shows that DNA extraction was unsuccessful from one out of four samples identified as 'Camellia' as well as the single sample of *Drimys* that was submitted for testing. Subsequent investigation of the optimal conditions for extraction from camellia suggest that failure of extraction is likely to be attributable to insufficient disruption of the material in the LFD buffer (data not shown). It is also possible that extraction failure is more likely for samples that are in poor condition at the time of testing, and this may account for at least some of the seven failed extractions from rhododendron, a host from which the majority of extractions were successful. Failure to detect host DNA from the *Drimys* sample could reflect either failure of extraction or failure of the COX assay to amplify *Drimys* DNA due to sequence differences. Because of the importance of the COX assay in the interpretation of negative results, isolation should be used for hosts which are not amplified by the COX assay.

The prevalence of *P. ramorum* in the samples submitted to the laboratory was comparable to that reported in the previous study by Kox et al. (2007) (45.9%). The use of *Phytophthora* spp. LFDs as a prescreen increases the effective 'prevalence' of *P. ramorum* in the group of samples reaching the laboratory since samples which do not contain any *Phytophthora* spp. are not submitted for testing. In this experiment, 79 of the 150 samples which tested positive by *Phytophthora* spp. LFD were found to contain *P. ramorum* by routine methods (52.7%). The other 71 samples presumably contained a different species of *Phytophthora*, or potentially a *Pythium* spp. with which the antibodies used in the LFD have been reported to cross-react (Lane et al. 2007). When testing at *P. ramorum* outbreak sites, the proportion of LFD positives attributable to the presence of *P. ramorum* (rather than another species of *Phytophthora*) may be somewhat higher than this. For example, in the study of Kox et al. (2007), 62 out of 68 rhododendron samples from known *P. ramorum* outbreak sites which tested positive for *Phytophthora* spp. by LFD were found to contain *P. ramorum* by isolation or real-time PCR. However, the predictive values of a test depend on the prevalence of the disease. For this reason, if a *P. ramorum*-specific test is to be used in conjunction with pre-screening by *Phytophthora* spp. LFDs, the predictive value of the of the *P. ramorum*-specific test depends not only on the prevalence of *P. ramorum* but also on the prevalence of other *Phytophthora* spp.. This suggests that the optimal testing strategy will be dependent on the source of the samples to be tested. For example, in scenarios such as the screening of nursery stock, the prevalence of species other than *P. ramorum* which are detected by the *Phytophthora* spp. LFD could result in a large number of LFD-positive

samples being submitted for testing which do not actually contain *P. ramorum*. In this case, the PPV of the laboratory test could be lower than might be assumed on the basis of assay sensitivity and specificity, and it may be desirable to confirm any positive results using another method. Conversely, when testing at a *P. ramorum* outbreak site, the proportion of positive *Phytophthora* spp. LFDs attributable to *P. ramorum* is likely to be considerably higher. The NPV of even a highly sensitive and specific test could be lower than presumed, and care should therefore be taken in the interpretation of negative results. Statutory requirements notwithstanding, where prescreening reduces the number of negative samples submitted for testing, confirmation of negative rather than positive results is likely to be more efficient and result in fewer aberrant calls.

ACKNOWLEDGMENTS

The authors wish to acknowledge funding from Defra's Plant Health Division and the Proof of Concept Fund of the InterAct Partnership.

CHAPTER 3

Published in Phytopathology, 2010

J.A. Tomlinson, M.J. Dickinson and N. Boonham. (2010) Rapid detection of *Phytophthora ramorum* and *P. kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. *Phytopathology*, 100, 143-149

Author contribution:

Project supervised by M. Dickinson and N. Boonham.

Data collected and analysed and manuscript prepared by J.A. Tomlinson.

Rapid detection of *Phytophthora ramorum* and *P. kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device

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ABSTRACT

A method for nucleic acid-based detection of pathogens in plant material has been developed which comprises a simple and rapid method for extracting DNA on the nitrocellulose membranes of lateral flow devices, loop-mediated isothermal amplification (LAMP) of target DNA using labelled primers and detection of the generically labelled amplification products by a sandwich immunoassay in a lateral flow device format. Each of these steps can be performed without specialist equipment and is suitable for on-site use, and a result can be obtained in just over an hour. A LAMP assay for the detection of plant DNA (cytochrome oxidase gene) can be used in conjunction with pathogen-specific assays to confirm negative results. The use of this method is demonstrated for the detection of *Phytophthora ramorum*, the causal agent of

sudden oak death and dieback / leaf blight in a range of tree, shrub and herbaceous species, and the recently described pathogen *P. kernoviae*.

INTRODUCTION

Efficient detection of pathogens in plant material is necessary for the timely implementation of eradication and containment measures to prevent or limit the spread of plant diseases that can have severe economic and sociological consequences. Plant pathogens can be detected using a range of methods, including examination of symptoms or pathogen morphology, antibody-based methods such as ELISA, and nucleic acid-based methods (Ward et al. 2004). PCR-based detection methods are often favoured for their sensitivity and specificity (Ward et al. 2004; Mumford et al. 2006).

However, in order for samples to be subjected to PCR-based testing they need to be sent to a laboratory with the necessary facilities. Significant advantages could be gained from moving testing closer to the site of sampling and thereby reducing the delay between taking a sample and obtaining a result, but the majority of nucleic acid-based pathogen detection methods are too complex and time consuming for reliable routine use outside the laboratory. In addition, PCR-based detection methods generally require the extraction of high quality nucleic acid from the sample material, and this step is often found to be a bottleneck in terms of the time and operator skill required (Mumford et al. 2006).

Methods for pathogen detection in the field, as well as being sufficiently sensitive and specific, should also be rapid and simple, with results that are easy to interpret, and should demand minimal equipment and facilities. In addition to these features, field-testing methods should ideally be inexpensive, and the components should be disposable or easily decontaminated. With these requirements in mind, PCR-based methods have a

number of drawbacks which limit the feasibility of their use in field conditions. In particular, PCR-based methods require relatively complex and expensive thermal cycling equipment, particularly for real-time PCR, in which fluorescence detection is performed concurrently with thermal cycling. Real-time PCR can be performed in the field using portable, ruggedized platforms such as the Cepheid SmartCycler or Idaho R.A.P.I.D. (Schaad et al. 2002; Mavrodieva et al. 2004; Tomlinson et al. 2005), but cost and complexity may limit the applications for which these platforms are appropriate.

In contrast to PCR, isothermal amplification methods avoid the use of thermal cycling equipment, allowing reactions to be incubated in a water bath or simple heated block (Gill and Ghaemi 2008). Loop-mediated isothermal amplification (LAMP) is an amplification method which uses two sets of primers (internal and external primers) and a DNA polymerase with strand displacing activity to produce amplification products containing loop regions to which further primers can bind, allowing amplification to continue without thermal cycling (Notomi et al. 2000; Nagamine et al. 2001). Amplification is accelerated by the use of an additional set of primers (loop primers) that bind to those loops which are of the incorrect orientation for the internal primers to bind (Nagamine et al. 2002). A high level of specificity results from the requirement for primers to bind to up to eight regions of the target sequence, and the efficient generation of large amounts of amplification product permits the use of novel product detection methods (Mori et al. 2001). The use of LAMP has previously been described for the detection of a range of plant pathogens (Fukuta et al. 2003a, b; Fukuta et al. 2004; Nie 2005; Tomlinson et al. 2007; Tomlinson and Boonham 2008; Varga and James 2006).

LAMP products can be detected by conventional agarose gel electrophoresis, by the use of spectrophotometric equipment to measure turbidity (Mori et al. 2004), in real-time using intercalating fluorescent dyes (Maeda et al. 2005), or by visual inspection of turbidity or colour changes (Mori et al. 2001; Iwamoto et al. 2003). While detection methods based on visual inspection have the advantage of requiring no equipment, assessment of colour or turbidity with the unaided eye is potentially subjective. Equipment-free methods for unambiguous detection of LAMP products would increase the feasibility of using LAMP for detection of phytopathogens outside the laboratory. One such method is the use of lateral flow devices (LFDs) for the detection of labels incorporated into the amplification products (Kiatpathomchai et al. 2008), a technique sometimes referred to as Nucleic Acid Lateral Flow (NALF).

Tests in an LFD format have a number of advantages for use in the field, and specific LFD immunoassays have been extremely successful in areas of point-of-care and on-site testing, including for the detection of plant pathogens (Danks and Barker 2000; Lane et al. 2007). However, the development of pathogen-specific immunoassays in LFD format requires the availability of suitable antibodies, the generation of which can be expensive and time consuming. Further to this, the resulting immunoassays may not be sufficiently sensitive for reliable use in the field or sufficiently specific to identify the pathogen to the required taxonomic level. Nevertheless, the success of LFD-based diagnostic tests is an indicator of the ease with which these devices can be used and their results interpreted. Familiarity with this

type of test could help to facilitate the adoption of nucleic acid-based detection methods in an LFD format for use by non-laboratory staff.

Phytophthora ramorum (Werres et al. 2001) is the causal agent of mortality of tanoak (*Lithocarpus densiflorus* (Hook. and Arn.) Rehd.) and *Quercus* spp. (sudden oak death) in forests on the west coast of the United States (Rizzo et al. 2002), and dieback and leaf blight in a wide range of plant species in Europe and elsewhere. *Phytophthora kernoviae* is a more recently described species (Brasier at el. 2005) discovered in 2003 as the causal agent of a disease (with symptoms similar to *P. ramorum*) on rhododendron and beech trees in southwest England. LFDs are available for detection of *Phytophthora* spp. (Lane et al. 2007), and these have been successfully deployed in the field, but since these devices detect all members of the genus *Phytophthora,* further testing is required to identify the pathogen to the species level. Although genus-level identification is adequate for some applications, species-specific tests are required in some circumstances; for example, to discriminate between non-notifiable species and notifiable species such as *P. ramorum* and *P. kernoviae*. Rapid methods have been developed for detection of *P. ramorum* in the field (Tomlinson et al. 2005; Tomlinson et al. 2007), although the use of thermal cycling equipment and the need for a suitably rapid DNA extraction method limit the use of these methods in field conditions.

The Food and Environment Research Agency has developed a method for the extraction of nucleic acid from LFDs (Danks and Boonham 2007). Amplifiable nucleic acid can be extracted from plant material in less than 5 minutes without the use of any equipment, making this method potentially suitable for use in the field. This paper describes a simplified method for the

detection of *P. ramorum* and *P. kernoviae* in infected plant material using an extremely rapid one-step DNA extraction method, followed by specific isothermal amplification, and detection of the amplification products in a generic and easily interpreted LFD format. The pathogen-specific assays are used in conjunction with an internal control assay for the detection of the cytochrome oxidase (COX) gene of the host plant, in order to confirm that DNA extraction was successful. A result can be obtained in just over 1 hour, with less than 10 minutes of hands-on time without the need for complex or expensive equipment.

MATERIALS AND METHODS

Phytophthora ramorum and *P. kernoviae* inoculation of plant material

Isolates of *P. ramorum* and *P. kernoviae* were grown on carrot piece agar (CPA) (Werres et al. 2001) for at least one week, then 0.5 cm^2 agar plugs were taken from the leading edge of colonies and used to inoculate wounded detached leaves of Rhododendron 'Cunningham's White', which were incubated at room temperature in a damp chamber for at least one week.

DNA extracts for characterization of LAMP specificity and sensitivity

Isolates of *Phytophthora* spp. were grown on semi-selective P_5ARP-(H) agar (Jeffers and Martin 1986) or CPA. DNA was extracted from 0.5 cm^2 plugs taken from the cultures using the NucleoSpin Plant kit (Machery-Nagel, Düren, Germany) following the manufacturer's protocol for fungi (Hughes et al. 2006b).

DNA was extracted from *Phytophthora*-inoculated and uninoculated plant material (approximately 0.5 g samples) using the CTAB-based method used by Suarez et al. (2005). DNA extracts were quantified by spectrophotometry and diluted as required in nuclease-free water.

Extraction of DNA using lateral flow devices

DNA was extracted using LFDs in a process consisting of disruption of plant material in an extraction buffer followed by application of an aliquot of the buffer containing disrupted material to the release pad of the LFD, allowing it to run along the device's nitrocellulose membrane (Danks and Boonham 2007). DNA on the LFD membrane can be amplified by adding a section of the membrane directly to a DNA amplification reaction, such as LAMP. LFDs for DNA extraction were purchased from Forsite Diagnostics Ltd (York, UK). Samples of leaf material (0.3 g) were placed in plastic bottles containing five steel ball bearings (5 mm diameter) and 5 ml of LFD Buffer C (Forsite Diagnostics Ltd), and vortexed or shaken vigorously for 90 seconds to disrupt the sample material. This method results in sufficient disruption of the plant material for the release of DNA without complete homogenisation of the sample (Danks and Boonham 2007). Seventy microlitres of Buffer C was transferred from the bottle to the release pad of the extraction LFD and allowed to flow across the membrane. The devices were allowed to dry at room temperature, typically for around 5 minutes, or in some cases for several hours. After this time, devices were stored in a sealed bag at room temperature.

LAMP primer design

LAMP primers for *P. ramorum* were as previously described (Tomlinson et al. 2007). New LAMP primers were designed to detect *P. kernoviae* (based on the ITS sequence of a range of *Phytophthora* species, as previously described by Hughes et al. (2006b), and an assay was designed for the detection of plant DNA based on cytochrome oxidase (COX) sequence (Weller et al. 2000). Primer design was carried out using the LAMP primer design software PrimerExplorer V3. Six LAMP primers (external primers F3 and B3, internal primers FIP and BIP, and loop primers F-Loop and B-Loop) were designed for each assay. For details of the principle of the LAMP method, see Notomi et al. (2000) and Section 1.5.4. Primers were synthesized by Sigma-Aldrich (Haverhill, UK); primer sequences are shown in Table 3.1.

Table 3.1. Primers used for loop-mediated isothermal amplification

Primer	Sequence (5′ -3′)
P.ram F3	CTAAAAAACTTTCCACGTGAAC
P.ram B3	CTTCATCGATGTGCGAGC
P.ram FIP	TCAAGCGCTCGCCATGATAGAGTCAAAACCCTTAGTTGGGGGCT
P.ram BIP	ACTTTTTAAACCCATTCCTAAATACTGAACATCCACTGCTGAAAGTTGC
P.ram F-Loop[1]	CGAAGCCAGCCGAACAGA
P.ram B-Loop[2]	GTGGGGACGAAAGTCTCTG
P.kern F3	TGTCGGCGACTAATTTCGTG
P.kern B3	CGCATTGTCCGAAAACAACA
P.kern FIP	GCAGATTGTTCGGCCGAAACCCTGAGGCGTTTTGGAGAGG
P.kern BIP	TTCCTTGCTTTGGCGTTTGCGCGCACACAAAGTTTCGTTCA
P.kern F-Loop[1]	CACTACCGCGAATCGAACC
P.kern B-Loop[2]	TGGTGTACCGTAGTAGTGTGTAGCT
COX F3	TATGGGAGCCGTTTTTGC
COX B3	AACTGCTAAGRGCATTCC
COX FIP	ATGGATTTGRCCTAAAGTTTCAGGGCAGGATTTCACTATTGGGT
COX BIP	TGCATTTCTTAGGGCTTTCGGATCCRGCGTAAGCATCTG
COX F-Loop[3]	ATGTCCGACCAAAGATTTTACC
COX B-Loop[2]	GTATGCCACGTCGCATTCC

[1]5′-labelled with digoxigenin (DIG) when used with LFD detection of LAMP products.

[2]5′-labelled with biotin when used with LFD detection of LAMP products.

[3]5′-labelled with fluorescein isothiocyanate (FITC) when used with LFD detection of LAMP products

Loop-mediated isothermal amplification

Extracted DNA (1 µl) was added to 24 µl of reaction mix, and negative controls containing nuclease-free water instead of DNA were included in each run. When LFDs were being tested, a section of the LFD membrane (approximately 5 mm x 1 mm) was added directly to the LAMP reaction mix. Sections were generally taken from the centre of the membrane, although it is not necessary to sample from any particular region of the membrane (Danks and Boonham 2007). The *P. ramorum* and *P. kernoviae* LAMP reaction mixes consisted of 0.32 U μl^{-1} *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), 1 x Thermopol buffer (New England Biolabs), 1.4 mM each dNTP, 6 mM $MgSO_4$ (including 2 mM in Thermopol buffer), 1.2 M betaine, 200 nM each external primer (F3 and B3), 2 µM each internal primer (FIP and BIP), and 1 µM each loop primer (F-Loop and B-Loop).

The plant cytochrome oxidase (COX) LAMP reaction mix consisted of 0.64 U μl^{-1} *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), 1 x Thermopol buffer, 1.4 mM each dNTP, 8 mM $MgSO_4$ (including 2 mM in Thermopol buffer), 0.8 M betaine, 400 nM each external primer (F3 and B3), 4 µM each internal primer (FIP and BIP), and 2 µM each loop primer (F-Loop and B-Loop).

Reactions were incubated at 65°C for 60 minutes, then at 80°C for 5 minutes to inactivate the *Bst* polymerase. Amplification products were visualised by gel electrophoresis: LAMP products consist of products of different lengths containing alternately inverted repeats of the target sequence, appearing as a ladder-like pattern when visualised on a gel (Notomi et al. 2000).

The COX LAMP assay was optimised using a range of concentrations of $MgSO_4$ (4 – 10 mM), betaine (0.8 – 1.6 M), primers (0.2 – 4 µM) and *Bst* polymerase (0.32 – 0.64 U μl^{-1}). Optimal conditions as described above were selected on the basis of the amount of product as assessed by gel electrophoresis (sub-optimal conditions often resulted in no amplification). For assay optimisation and characterization, reactions were carried out in duplicate.

LAMP using labelled primers

LAMP was carried out using labelled primers to allow detection of amplification products by LFD. For each assay, one loop primer (B-loop) was labelled at the 5′ end with biotin and the other loop primer (F-loop) was labelled at the 5′ end with either digoxigenin (DIG) (*P. ramorum* and *P. kernoviae* assays) or fluorescein isothiocyanate (FITC) (COX assay). Labelled primers were synthesized by Eurofins MWG (Ebersberg, Germany).

Detection of labelled LAMP products by LFD

LFDs work by immunochromatography. The sample (labelled LAMP product in this case) is applied to the release pad of the device which contains coloured latex coated with a reagent which specifically binds to the target molecule. The target-latex complex flows through the membrane to a test line containing a reagent which also binds to the target-latex complex, forming a visible line if the target is present. In this case, the reagent at the test line binds to one of the labels incorporated into the LAMP product (either DIG or FITC) and the latex binds to the other label (biotin). A test line is therefore only formed when both labels are incorporated into the amplification product; no

test line is formed for negative reactions in which only unincorporated primers are present. The devices also have a control line containing a reagent which binds directly to the coated latex, such that a negative result is indicated by a single line (showing that the device has run successfully) and a positive results is indicated by two lines (Figure 3.1). Devices for the detection of labelled LAMP products were purchased from Forsite Diagnostics. After amplification, the labelled LAMP reactions were diluted 1 in 500 in LFD Buffer C (Forsite Diagnostics), then approximately 70 µl of diluted reaction was applied to the release pad of the device. The DIG/biotin devices and the FITC/biotin devices contained red and blue latex, respectively (Figure 3.1).

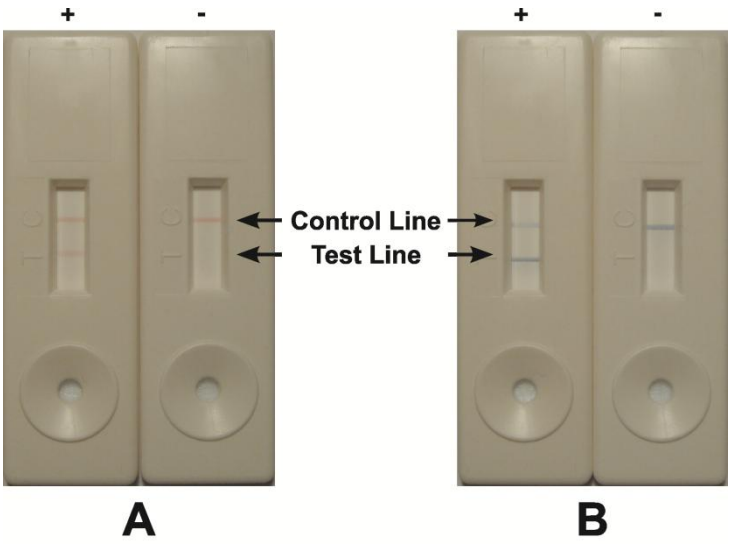

Figure 3.1. Lateral flow devices (LFDs) for the detection of LAMP products labelled with digoxigenin (DIG) and biotin (**A**) or fluorescein isothiocyanate (FITC) and biotin (**B**), showing positive (+) and negative (-) results.

Multiplex LAMP

LAMP reactions for multiplex detection of either *P. ramorum* and COX or *P. kernoviae* and COX were carried out using a reaction mix consisting of 0.32 U μl^{-1} Bst DNA polymerase (New England Biolabs, Ipswich, MA), 1 x Thermopol buffer, 1.4 mM each dNTP, 8 mM $MgSO_4$ (including 2 mM in Thermopol buffer), 0.8 M betaine, 400 nM each COX external primer, 4 µM each COX internal primer, 2 µM each COX loop primer, 200 nM each pathogen external primer, 2 µM each pathogen internal primer, and 1 µM each pathogen loop primer.

Field samples

A small number of rhododendron samples were tested that had been collected by Defra's Plant Health and Seeds Inspectorate (PHSI) as part of ongoing surveillance for *P. ramorum* and *P. kernoviae*. Each sample was dispatched to the laboratory in a sealed plastic bag containing a small piece of damp tissue. On receipt in the laboratory the material was examined for the presence of typical symptoms, and samples were taken from the leading edge of any identified lesions for routine diagnositic testing (isolation on P_5ARP-(H) or detection by TaqMan real-time PCR) (Hughes et al. 2006b). Duplicate samples were taken for extraction by LFD and testing by simplex LAMP using labelled primers, as described above.

RESULTS

P. kernoviae and COX LAMP assays

When visualised by agarose gel electrophoresis, the products of the *P. kernoviae* and COX assays showed the ladder-like pattern expected for LAMP products (Figure 3.2). Amplification was not observed in the negative control reactions.

The lowest amount of *P. kernoviae* DNA to be consistently amplified using the *P. kernoviae* LAMP assay was approximately 17 pg (Figure 3.2). This is comparable to the level of sensitivity observed for the *P. ramorum* LAMP assay developed previously (Tomlinson et al. 2007). DNA extracts from cultures of other *Phytophthora* species including *P. boehmeriae*, *P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. citricola*, and *P. ramorum* were also tested and none of these species were amplified by the *P. kernoviae* LAMP assay (data not shown).

The lowest amount of rhododendron DNA to be consistently amplified using the COX LAMP assay was approximately 9 ng (Figure 3.2). Amplification was also sometimes observed with 900 pg rhododendron DNA (as in Figure 3.2), indicating that this is close to the limit of detection for this assay. The COX LAMP assay was also used to amplify DNA extracted from viburnum and camellia, two other commonly encountered hosts of *P. ramorum* in the UK (Figure 3.2).

Labelled LAMP and LFD detection of labelled LAMP products

The use of labelled primers did not adversely affect any of the assays tested as determined by gel electrophoresis of the products (data not shown). DIG/biotin- and FITC/biotin-labelled LAMP products were run on DIG or FITC LFDs as appropriate, and the LFD results were consistent with the results of gel electrophoresis (Figure 3.3). LFD test lines developed in less than 5 minutes, and control lines were observed for all devices.

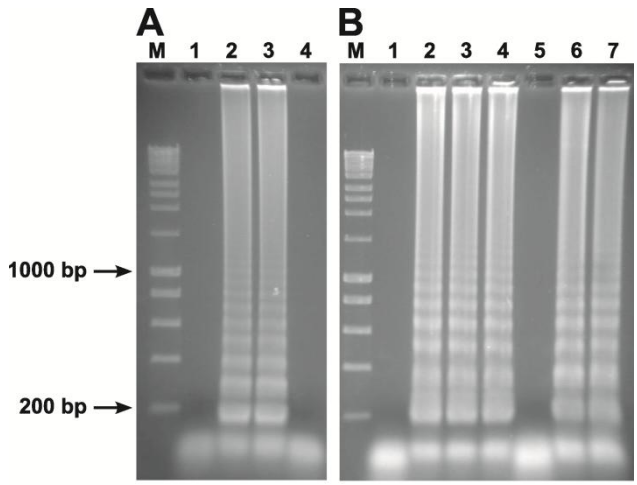

Figure 3.2. Loop-mediated isothermal amplification (LAMP) for the detection of *Phytophthora kernoviae* and plant cytochrome oxidase (COX) gene. **A.** Dilutions of *P. kernoviae* DNA were amplified by *P. kernoviae* LAMP and the products were visualised by gel electrophoresis. M: marker (HyperLadder I (New England Biolabs)); lane 1: no template control; lane 2: 170 pg *P. kernoviae* DNA; lane 3: 17 pg *P. kernoviae* DNA; lane 4: 1.7 pg *P. kernoviae* DNA. **B.** Dilutions of rhododendron DNA, and DNA extracted from viburnum and camellia were amplified by COX LAMP and the products were visualised by gel electrophoresis. M: marker; lane 1: no template control; lane 2: 90 ng rhododendron DNA; lane 3: 9 ng rhododendron DNA; lane 4: 900 pg rhododendron DNA; lane 5: 90 pg rhododendron DNA; lane 6: viburnum DNA (approximately 100 ng); lane 7: camellia DNA (approximately 100 ng).

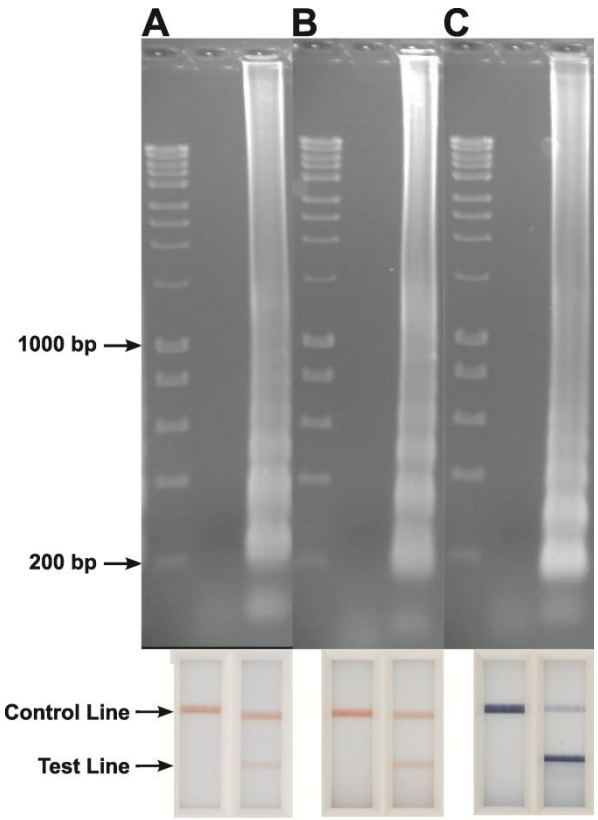

Figure 3.3. Detection of labelled LAMP products by gel electrophoresis (top) and lateral flow device (bottom). The same labelled LAMP products shown in the gel electrophoresis pictures were diluted 1 in 500 and run on the devices shown below each lane. **A.** *Phytophthora ramorum* LAMP assay (DIG and biotin labels). M: marker (HyperLadder I (New England Biolabs)); lane 1: no template control; lane 2: *P. ramorum* DNA. **B.** *P. kernoviae* LAMP assay (DIG and biotin labels). M: marker; lane 1: no template control; lane 2: *P. kernoviae* DNA. **C.** Plant cytochrome oxidase (COX) LAMP assay (FITC and biotin labels). M: marker; lane 1: no template control; lane 2: rhododendron DNA.

Multiplex LAMP

The *P. ramorum* LAMP assay was used in multiplex with the COX LAMP assay to test CTAB DNA extracts from healthy and *P. ramorum*-infected rhododendron and an extract from *P. ramorum* culture. The multiplex products were run on DIG and FITC LFDs, demonstrating the detection of single products (*P. ramorum* or COX) and mixed products (Figure 3.4a).

The *P. kernoviae* LAMP assay was also used in multiplex with the COX assay to test extracts from *P. kernoviae* culture and healthy and *P. kernoviae*-infected rhododendron. In this case, the infected rhododendron failed to generate a positive COX result (Figure 3.4b), although *P. kernoviae* was amplified from this sample. Pathogen DNA and plant DNA was amplified when the same extract was tested with the assays in separate tubes (data not shown). Subsequent experiments testing different ratios of *P. kernoviae* and plant DNA suggested that these assays used in multiplex could simultaneously amplify both targets unless one of the targets was present in excess (data not shown). Since the ratio of *Phytophthora* DNA to host DNA could vary considerably between samples of infected material, it is preferable to use the host and pathogen assays in separate tubes to avoid the situation in which a low level of pathogen is not detected due to the presence of a large amount of plant DNA, or the potentially less serious situation where the presence of a large amount of pathogen DNA results in failure to amplify the plant DNA (as observed in Figure 3.4).

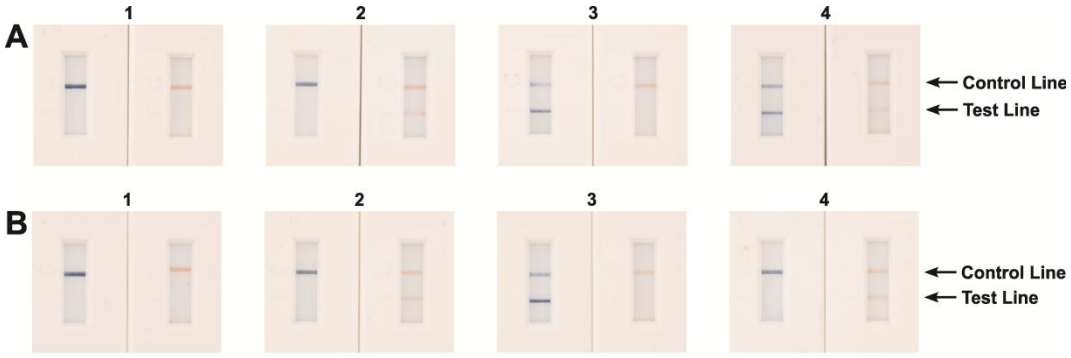

Figure 3.4. Multiplex LAMP. *Phytophthora ramorum* (**A**) and *P. kernoviae* (**B**) LAMP assays (using DIG and biotin labelled primers) were used in multiplex with the plant cytochrome oxidase (COX) LAMP assay (using FITC and biotin labelled primers) to test DNA extracted from cultures of *P. ramorum* and *P. kernoviae* and healthy and infected rhododendron leaves. 1: no template control; 2: culture; 3: healthy rhododendron; 4: infected rhododendron. FITC/biotin devices shown on left (blue), DIG/biotin devices shown on right (red).

LFD extraction method

The ability of the detection method comprising LFD DNA extraction followed by (simplex) LAMP using labelled primers and analysis of the LAMP products using generic DIG and FITC LFDs was examined in terms of the ability to detect *P. ramorum* or *P. kernoviae* in infected rhododendron leaves. Necrotic material from inoculated leaves of rhododendron 'Cunningham's White' was mixed with healthy rhododendron leaf to produce samples with a total weight of 0.3 g containing 10% necrotic tissue by weight, and DNA was extracted by vortexing or vigorous shaking with ball bearings in LFD Buffer C for 90 seconds before application to LFDs. No difference was observed between samples disrupted by vortexing and by manual shaking. The LFDs were tested by LAMP either for *P. ramorum* and plant DNA (COX), or for *P. kernoviae* and COX, depending on the pathogen with which the samples had been inoculated. Typical results are shown in Figure 3.5: positive COX results were obtained for both infected and non-infected samples, *P. ramorum* was detected in the *P. ramorum*-infected material, and *P. kernoviae* was detected in the *P. kernoviae*-infected material. Table 3.2 shows the results for replicate samples of healthy and inoculated rhododendron tested in this way. The same results were obtained for 10 replicate samples of healthy rhododendron and 10 replicate samples of *P. kernoviae*-inoculated rhododendron. Seven out of 10 samples containing 10% *P. ramorum*-infected rhododendron tested positive for *P. ramorum*, and all tested positive for COX. This suggests that the amount of *P. ramorum* DNA extracted from these samples was close to the limit of detection for this assay. Ten out of 10

samples containing 20% *P. ramorum*-infected rhododendron were positive for both *P. ramorum* and COX.

In addition to testing artificially inoculated rhododendron, a small number of naturally infected samples collected in the field were tested using the same method (Table 3.3). The results for these samples concurred with those obtained by routine laboratory testing (isolation on P_5ARP-(H) or detection by TaqMan real-time PCR) (Hughes et al. 2006b).

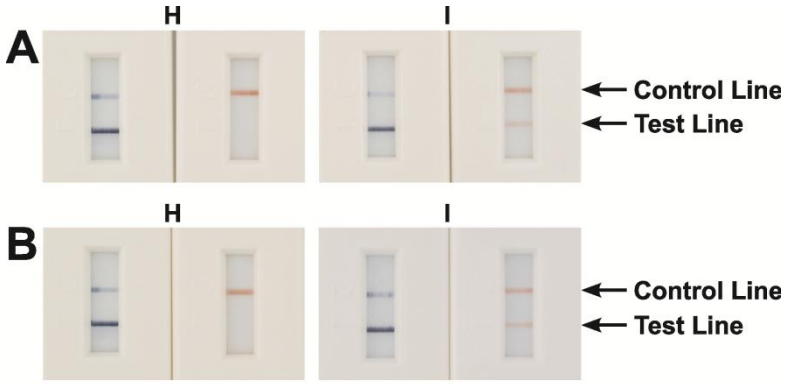

Figure 3.5. Detection of *Phytophthora ramorum* (**A**) and *P. kernoviae* (**B**) in infected rhododendron leaf. DNA was extracted using lateral flow devices (LFDs) from samples of either healthy rhododendron leaf, or rhododendron leaf mixed with *P. ramorum*- or *P. kernoviae*-infected rhododendron leaf to give 10% infected material by weight. LFDs were tested by labelled LAMP (not in multiplex) for COX (FITC and biotin labels) and either *P. ramorum* or *P. kernoviae* (DIG and biotin labels), respectively. The amplification products were applied to DIG/biotin and FITC/biotin LFDs. H: healthy rhododendron; I: infected rhododendron (10% infected material by weight). FITC/biotin devices shown on left (blue), DIG/biotin devices shown on right (red).

Table 3.2. Summary of results for samples of healthy and artificially inoculated rhododendron tested by LFD DNA extraction followed by labelled LAMP and LFD detection of LAMP products.

	LAMP result with LFD detection of product (number of samples positive/number of samples tested)		
Sample type	COX	*P. ramorum*	*P. kernoviae*
Healthy rhododendron	10/10	0/10	0/10
P. ramorum (10%)[1]	10/10	7/10	n/t
P. ramorum (20%)[1]	10/10	10/10	n/t
P. kernoviae (10%)[1]	10/10	n/t	10/10

n/t: not tested

[1]Percentage infected material by weight (total sample weight 0.3 g).

Table 3.3. Summary of results for samples of naturally infected rhododendron tested by LFD DNA extraction followed by labelled LAMP and LFD detection of products.

		LAMP result with LFD detection of product		
Sample	Laboratory diagnosis[1]	COX	*P. ramorum*	*P. kernoviae*
1	*P. ramorum*	positive	positive	negative
2	*P. ramorum*	positive	positive	negative
3	*P. kernoviae*	positive	negative	positive
4	negative	positive	negative	negative
5	negative	positive	negative	negative

[1]Result of laboratory testing for *P. ramorum* and *P. kernoviae* by isolation on semi-selective media or TaqMan real-time PCR.

DISCUSSION

The pathogen detection method described here attempts to address some of the main requirements of on-site testing. The workflow is relatively simple in comparison with many existing nucleic acid-based detection methods and generates results in an easily interpreted format in just over 1 hour, including DNA extraction. In addition, the LAMP reaction mix can be prepared in advance and lyophilized to allow room temperature storage. Each of the three steps (manual shaking to disrupt the sample before application onto the DNA extraction LFD, placing a section of LFD membrane into pre-prepared LAMP reaction mix and incubation in a heated block or water bath, and dilution of the LAMP reaction and application onto the detection LFDs) is sufficiently simple to potentially allow this method to be performed outside a conventional laboratory facility without extensive prior training. Previous methods for nucleic acid-based detection of plant pathogens in the field have sought to transfer established laboratory methods (for example, magnetic bead-based DNA extraction followed by real-time PCR) into a non-laboratory environment (Tomlinson et al. 2005; Hughes et al. 2006a). However, methods that more specifically address the requirements for fewer steps using minimal equipment have the potential to be adopted in a wider range of settings. This method also compares favourably with existing methods in terms of the costs of DNA extraction and LAMP reagents. The cost of consumables required for LFD extraction of DNA (LFDs and buffer bottles) is similar to or less than the cost of commonly used extraction kits based on spin columns or magnetic beads, without taking into account the staff time and equipment required to use these kits. Primers labelled with DIG, FITC and biotin can cost several times

more per batch than un-modified oligonucleotides (equivalent to several pence per reaction) and typically cost around half the price (per reaction) than the fluorescently labelled oligonucleotide probes required for real-time PCR. In addition, the internal primers FIP and BIP require HPLC purification due to their length. The per-reaction cost of labelled LAMP is therefore broadly comparable to real-time PCR. A typical small lab carrying out nucleic acid extraction by conventional methods followed by PCR and/or real-time PCR might be expected to be equipped with some or all of the following: waterbath or heated block, centrifuge, vortexor, pipettors, refridgerated and frozen storage for reagents and samples, thermal cycler, equipment for gel electrophoresis, and real-time PCR instrument. Of these, however, only a water bath or heated block and pipettors (as well as a scalpel or similar instrument for cutting the DNA extraction LFD membranes) are required to carry out the method described in this article.

LFDs can be valuable tools for on-site pathogen detection, and the speed and simplicity of tests in this format have promoted adoption of this technology (Danks and Barker 2000; Lane et al. 2007). However, some pathogen detection LFDs have limitations in terms of their specificity or sensitivity. The pathogen detection method that we describe could be used in conjunction with existing LFD immunoassays to mitigate these limitations. For example, labelled LAMP could be used to increase the specificity of pathogen detection in cases where it has not been possible to produce sufficiently specific antibodies. Species-specific antibodies are not available for *P. ramorum* or *P. kernoviae* but LFDs are currently available that detect all species of the genus *Phytophthora* (Lane et al. 2007). The labelled LAMP-

LFD method could be used to test any positive devices to determine whether the *Phytophthora* detected by the device is a particular species of interest (for example, *P. ramorum* or *P. kernoviae*, or any species for which a LAMP assay had been designed).For other pathogens, conventional LFDs may be available that are specific but insufficiently sensitive to detect the target pathogen in some samples. The labelled LAMP-LFD method could be used to test any samples that were negative by conventional LFD, in order to detect pathogen below the detection threshold of the initial test. Finally, in applications where no LFDs are currently available, the development of a LAMP assay for the pathogen of interest could be considerably less costly and time consuming than the development of target-specific antibodies.

A particular hurdle for the development of field testing methods for some plant pathogens is the requirement for the extraction of high quality nucleic acid. Extraction methods selected for use in the laboratory may be favoured for their low cost, high throughput, or amenability to automation. However, methods which have been developed with these requirements in mind are unlikely to be suitable for use outside the laboratory. Our previously described method for extraction of DNA from *P. ramorum*-infected plants using magnetic beads and a PickPen device (Bio-Nobile, Turku, Finland) could be completed in approximately 30 minutes and involved a number of pipetting steps (Tomlinson et al. 2005). While this method was more rapid and required less equipment than many conventional laboratory-based methods (which often require multiple incubation and centrifugation steps or the use of organic solvents), the feasibility of using this method in some non-laboratory situations is limited. In comparison, the LFD nucleic acid extraction method is completed

in less than five minutes and the samples can be disrupted by manual shaking. After extraction has been carried out in the field, the devices can be tested at the point of sampling, but because DNA is stable on the LFD membrane at room temperature (Danks and Boonham 2007), it is also possible to return the devices to a laboratory for testing. This approach may be preferable to moving potentially infected plant material away from the site of sampling, and also expedites testing at the laboratory since the devices can be tested directly (for example, by real-time PCR) without further processing.

The final step in the workflow described in this paper is the detection of labelled LAMP products using generic LFDs. The use of LFDs has been described for detection of the products of various nucleic acid amplification methods (Deborggraeve et al. 2006; Carter and Cary 2007; Kiatpathomchai et al. 2008; Srisala et al. 2008). In some cases the product is detected through the use of amplicon-specific capture and/or detector probes (Deborggraeve et al. 2006; Carter and Cary 2007). However, since these devices contain amplicon-specific oligonucleotides, it would be necessary to manufacture new devices for each target. The incorporation of labels which can be detected immunologically allows the use of generic devices for multiple applications, since the same labels can be incorporated in different assays (Mens et al. 2008). Incorporation of two labels allows the product to be detected in a sandwich format: generally, one label is incorporated into the amplification product using a labelled primer and the second label is incorporated using a labelled detector probe which hybridises to the amplification product (Deborggraeve et al. 2006; Kiatpathomchai et al. 2008; Srisala et al. 2008). The detector probe is intended to ensure specificity of detection, since the

amplicon must contain a sequence to which the detector probe will hybridise in order to produce a positive result. However, the amplification mechanism of LAMP confers inherent specificity because eight regions of the target sequence must be recognized for amplification to occur. We found that by incorporating the second label into the amplification product using a second labelled primer the required degree of specificity could be achieved without the need for any additional reagents.

Unlike gel electrophoresis, the use of intercalating dyes, and observation or measurement of turbidity, the LFD detection approach allows the resolution of mixed products generated by multiplex assays by using differently labelled primers in each assay. The plant COX assay was designed to assist in the interpretation of negative results by indicating the success or failure of DNA extraction, and the use of this assay is described here in multiplex with the pathogen-specific assays (Figure 3.4) as well as in simplex (Figures 3.2, 3.3 and 3.5). Whether the pathogen-specific and plant control assays should be used individually or in multiplex is likely to depend on the target pathogen and the plant matrices to be tested. The challenge of optimising a robust multiplex reaction is compounded in the case of LAMP by the use of 6 primers for each assay. For applications where the ratio of pathogen DNA to plant DNA varies widely between samples it is preferable to use the pathogen and COX assays in separate tubes to avoid failure to detect low levels of pathogen in the presence of large amounts of plant DNA. A pathogen/plant multiplex assay could be more easily optimised for applications where the ratio of pathogen to plant DNA is more predictable.

In order to detect LAMP products on the LFDs used here it was necessary to dilute the products approximately 1 in 500 before applying them to the device. However, as has been discussed elsewhere (Mori et al. 2006; Tomlinson et al. 2007), opening the reaction tubes after amplification increases the risk of cross-contamination with previously amplified product (although once diluted the products pose less of a risk). The avoidance of contamination is likely to be the major challenge in the adoption of LAMP for routine use, especially outside the laboratory. A closed tube method for the detection of labelled products of isothermal helicase-dependant amplification has been described (Goldmeyer et al. 2008). Modifications to the reported method to avoid any handling of the undiluted amplification products would help to make the method more robust.

Validation in comparison with established methods is required before the method described in this paper could be used for a particular application. While the sensitivity of a LAMP assay can approach that of real-time PCR, the LFD extraction method is somewhat less efficient than more laborious conventional methods. Nevertheless, the method described here was sufficiently sensitive for testing symptomatic *Phytophthora*-infected rhododendron (mixed 1 in 10 or 1 in 5 with non-infected material), and the feasibility of testing naturally infected rhododendron has been demonstrated for a small number of samples. Further optimisation of the DNA extraction method may be required for different sample types containing different levels of pathogen.

ACKNOWLEDGMENTS

This work was funded by Plant Health Division, Defra, project PH0424.

CHAPTER 4

Published in Letters in Applied Microbiology, 2010

J.A. Tomlinson, M.J. Dickinson and N. Boonham. (2001) Detection of *Botrytis cinerea* by loop-mediated isothermal amplification. *Letters in Applied Microbiology*, 51, 650-657

Author contribution:

Project supervised by M. Dickinson and N. Boonham.

Data collected and analysed and manuscript prepared by J.A. Tomlinson.

Detection of *Botrytis cinerea* by loop-mediated isothermal amplification

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ABSTRACT

Aims: To develop a sensitive, rapid and simple method for detection of *Botrytis cinerea* based on loop-mediated isothermal amplification (LAMP) that would be suitable for use outside a conventional laboratory setting.

Methods and Results: A LAMP assay was designed based on the intergenic spacer (IGS) of the *B. cinerea* nuclear ribosomal DNA (rDNA). The resulting assay was characterized in terms of sensitivity and specificity using DNA extracted from cultures. The assay consistently amplified 65 pg *B. cinerea* DNA. No cross-reactivity was observed with a range of other fungal pathogens, with the exception of the closely related species *B. pelargonii*. Use of a novel real-time LAMP platform (the OptiGene Genie I) allowed detection of *B. cinerea* in infected rose petals, with amplification occurring in <15 minutes.

Conclusions: The LAMP assay that was developed is suitable for rapid detection of *B. cinerea* in infected plant material.

Significance and Impact of the Study: The LAMP method combines the sensitivity and specificity of nucleic acid-based methods with simplified equipment and a reduced reaction time. These features make the method potentially suitable for on-site use, where the results of testing could help to inform decisions regarding the storage and processing of commodities affected by *B. cinerea*, such as cut flowers, fruit and vegetables.

Keywords: *Botrytis*; grey mould; isothermal amplification; quantitative detection; rapid methods.

INTRODUCTION

Botrytis cinerea is the causal agent of grey mould in a wide range of plant species including many crops of economic importance (Williamson et al. 2007). Often present as a latent infection, this ubiquitous pathogen has the potential to cause damaging symptoms on commodities such as fruit, vegetables and cut flowers following a period of quiescence of unpredictable duration. Immunological and nucleic acid-based methods have been developed for the detection of *B. cinerea* (Bossi and Dewey 1992; Rigotti et al. 2002; Dewey and Meyer 2004; Suarez et al. 2005; Spotts et al. 2008; Celik et al. 2009). For some applications (for example, detection of *Botrytis* in grape juice) testing is carried out at the end of the decay process; however, for other applications testing is necessary in the early stages of infection, such as prior to storage of fruit or other commodities (Spotts et al. 2009). Nucleic acid-based methods have been successful in the detection of *B. cinerea* in presymptomatic infection (Suarez et al. 2005; Celik et al. 2009). Further to the requirement for high sensitivity, some applications also require the ability to detect *B. cinerea* quantitatively, and this has been achieved using both antibody- and nucleic acid-based methods (Meyer et al. 2000; Dewey and Meyer 2004; Mehli et al. 2005; Suarez et al. 2005; Celik et al. 2009). Knowledge of pathogen levels can help to inform production and storage decisions (Spotts et al. 2008), and quantitative methods also allow periods of active colonization to be distinguished from periods of quiescence (Cadle-Davidson 2008).

Nucleic acid-based methods for detection of *B. cinerea* are most commonly based on PCR (Rigotti et al. 2002; Brouwer et al. 2003; Gachon and Saindrenan 2004; Mehli et al. 2005; Suarez et al. 2005; Cadle-Davidson

2008; Celik et al. 2009). In particular, real-time PCR methods can be highly sensitive, with the potential for accurate quantification. These methods can therefore be valuable tools for investigating latent infection and the early stages of disease. However, real-time PCR-based methods can be time consuming, and the complexity of the equipment required to perform them restricts routine use of these methods to laboratory facilities. In some circumstances it is preferable to perform testing at the site of sampling, for example, in the field or within the production chain. PCR-based methods generally do not approach the speed and simplicity of field-portable antibody-based methods such as lateral flow devices (LFDs) (Lane et al. 2007) or simplified tube-format ELISA (Dewey and Meyer 2004).

Methods for isothermal amplification of nucleic acid do not require thermal-cycling equipment and therefore have the potential to be more suitable for on-site use than PCR-based methods. Loop-mediated isothermal amplification (LAMP) is a method which uses four or six specially designed primers and a DNA polymerase with strand displacing activity to generate amplification products which contain single-stranded loops, allowing primers to bind without the need for repeated cycles of thermal denaturation (Notomi et al. 2000; Nagamine et al. 2001). Briefly, forward and reverse internal primers, each consisting of a 3′ region which binds to the target and a 5′ region which is complementary to the target, interact with upstream external primers, resulting in the displacement of strands containing self-complementary regions which form stem-loop structures. The single-stranded loop regions act as primer binding sites. An additional pair of primers (loop primers) can be used to accelerate amplification by binding to those loops which are of the incorrect

orientation to bind the internal primers (Nagamine et al. 2002). A high level of specificity is conferred by the requirement for primers to bind to eight different regions of the target sequence, and LAMP assays have been reported for which sensitivity approaches that of real-time PCR (Notomi et al. 2000). LAMP products can be detected at the end-point of amplification by gel electrophoresis, or alternatively by observation of precipitated magnesium pyrophosphate generated as a by-product of amplification (Mori et al. 2001); by visual inspection after addition of colour-changing reagents (Iwamoto et al. 2003; Goto et al. 2009); or using LFDs to detect labels incorporated into the products during or after amplification (Kiatpathomchai et al. 2008; Tomlinson et al. 2010c). The generation of LAMP products can also be monitored in real-time (by measurement of turbidity or fluorescence), allowing quantitative detection of the target (Mori et al. 2004; Maeda et al. 2005). The Genie I instrument (OptiGene, Horsham, UK) is a portable, low-power platform for real-time fluorescence monitoring of isothermal amplification methods such as LAMP which is suitable for on-site use.

This paper describes the design and optimisation of a LAMP assay for detection of *B. cinerea* in infected plant material. This method was compared to existing laboratory and field methods (*B. cinerea* real-time PCR and *Botrytis* lateral flow device) for detection of *B. cinerea* in presymptomatic inoculated rose petals. The LAMP assay was also used in a real-time detection format on the Genie I instrument.

MATERIALS AND METHODS

Inoculation of plant material

Detached rose petals and pelargonium leaves were surface sterilized in 70% ethanol for 30 s, 5% bleach for 2 minutes, and finally in sterile distilled water for 5 minutes. A suspension of *B. cinerea* conidia was prepared from a 2-week -old culture (Fera culture collection reference cc1508,) on potato dextrose agar (PDA) using the method described by Suarez et al. (2005). Individual rose petals and pelargonium leaf discs (1 cm diameter) were inoculated with a 4 -µl aliquot of conidial suspension containing approximately 50 conidia μl^{-1}. The inoculated petals and leaf discs were incubated at room temperature in sealed dishes containing damp paper. To provide DNA for initial testing and sensitivity testing, petals and leaf discs were removed after approximately 3 days (after development of symptoms) for DNA extraction. In a separate experiment to compare detection methods, rose petals were incubated for up to 55 hours after inoculation and petals removed and tested at intervals during incubation.

DNA extraction from cultures and plant material

DNA was extracted from approximately 200 mg mycelium taken from the surface of cultures grown for at least 2 weeks on PDA. Extraction from non-*Botrytis* cultures was carried out using a NucleoSpin Plant kit (Machery-Nagel, Düren, Germany) following the manufacturer's protocol for fungi. Extractions from *Botrytis* spp. were carried out using a CTAB (cetyltrimethylammonium bromide) method. Mycelium was scraped from the surface of a 1- to 2- week-old culture on PDA and homogenized in a mortar

and pestle with 1.5 ml CTAB buffer, then processed using the method described by Suarez et al. (2005). DNA extracted from cultures was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA). Extracts prepared using the NucleoSpin method had concentrations ranging from 1 to 25 ng μl^{-1} and those prepared using the CTAB method had concentrations ranging from 50 to 150 ng μl^{-1}. A tenfold dilution series was prepared from DNA extracted from mycelium of *B. cinerea* (Fera cc1508) with concentrations ranging from 65 ng μl^{-1} to 650 fg μl^{-1}.

DNA was extracted from individual rose petals or pooled samples of four pelargonium leaf discs using the CTAB method described by Suarez et al. (2005). Ten-fold dilution series were prepared from extracts from symptomatic inoculated rose petals and pelargonium leaf discs incubated for 3 days, as described earlier. The concentration of *B. cinerea* DNA in dilutions of these extracts was estimated by quantitative real-time PCR using the method of Suarez et al. (2005) and the real-time PCR conditions described below. The concentrations in the 10^{-2} dilutions were approximately 14 ng μl^{-1} and 3 ng μl^{-1} for rose and pelargonium, respectively (data not shown).

LAMP primer design

Six LAMP primers (external primers F3 and B3, internal primers FIP and BIP, and loop primers F-Loop and B-Loop) were designed using the LAMP primer design software Primer Explorer 3. Primer sequences are shown in Figure 4.1. The primers were synthesized by Sigma-Aldrich (Haverhill, UK). The assay was designed to target the intergenic spacer (IGS) of the *B. cinerea* nuclear ribosomal DNA (rDNA) sequence. This region was selected

for a previously designed TaqMan real-time PCR assay (Suarez et al. 2005) to target mismatches between *B. cinerea* and non-target species, including the closely related species *B. fabae* and the less closely related species *B. tulipae*, *B. narcissicola* and *B. elliptica*. Sequence data for the IGS region has been published for relatively few *Botrytis* species, so the molecular phylogeny of Staats et al. (2005) was used to select the three most closely related species for specificity testing: *B. calthae*, *B. fabae* and *B. pelargonii*.

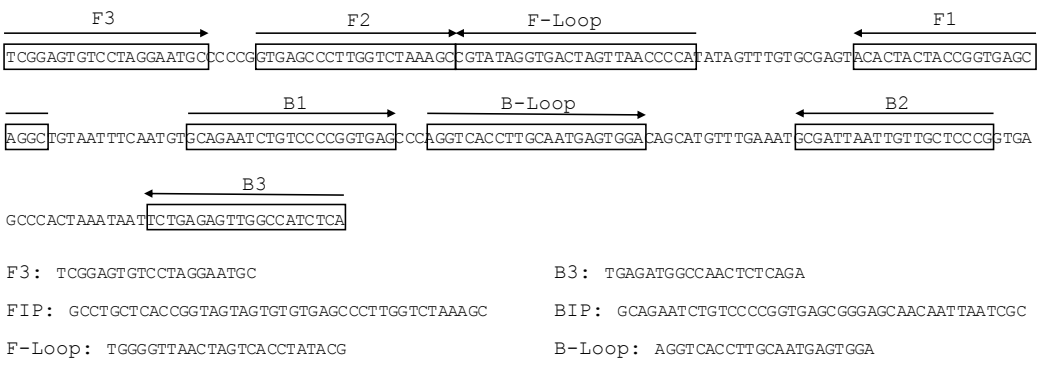

Figure 4.1. Primer design for loop-mediated isothermal amplification assay. Internal primer FIP is composed of the complementary sequence to F1 plus the sequence F2; internal primer BIP is composed of the sequence B1 plus the complementary sequence to B2. *Botrytis cinerea* isolate SAS56 sequence accession number: AM233400.

LAMP

LAMP was carried out by adding 1 µl DNA extracted from culture or inoculated rose or pelargonium to 24 µl of master mix. Negative controls containing nuclease-free water instead of DNA were included in each run. For assay development and characterisation, amplified products were visualized by staining with ethidium bromide following electrophoresis on 1.4% agarose gels. In order to optimise the LAMP reaction, the concentration of betaine was varied from 1.2 to 1.6 M, and the concentration of $MgSO_4$ was varied from 4 to 8 mM. Optimal concentrations were selected on the basis of the amount of product generated. The optimised LAMP master mix consisted of 0.32 U μl^{-1} *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), 1 x Thermopol buffer (supplied with *Bst* polymerase), 1.4 mM each dNTP, 6 mM $MgSO_4$ (including 2 mM in the Thermopol buffer), 1.2 M betaine, 200 nM each external primer (F3 and B3), 2 µM each internal primer (FIP and BIP), and 1 µM each loop primer (F-Loop and B-Loop). Reactions were incubated at 65°C for 60 minute, then at 80°C for 5 minutes to inactivate the *Bst* polymerase. All reactions were carried out in duplicate, and reach run was repeated at least once.

In order to characterise the sensitivity of the LAMP primers, DNA extracted from *B. cinerea* was tested in a tenfold dilution series ranging from 65 ng μl^{-1} to 650 fg μl^{-1}. In order to investigate the specificity of the LAMP assay, DNA extracts from cultures of the following non-target organisms were tested: *Alternaria brassicola* (Fera cc805), *B. narcissicola* (Fera cc771), *Colletotricum spp.* (Fera cc1433), *Cladosporium herbarum* (Fera cc1103), *Fusarium avenaceum* (Fera cc121), *Penicillium expansum* (Fera cc1102) and

Sclerotinia sclerotiorum (Fera cc284) obtained from the Food and Environment Research Agency culture collection; and *B. calthae* (MUCL1089), *B. fabae* (MUCL98) and *B. pelargonii* (MUCL1152) obtained from the Belgian Coordinated Collection of Microorganisms (BCCM/MUCL, Louvain-la-Neuve, Belgium).

TaqMan real-time PCR

The primers and probes used for TaqMan real-time PCR were described by Suarez et al. (2005): forward primer Bc3F 5′-GCTGTAA TTTCAATGTGCAGAATCC-3′; reverse primer Bc3R: 5′-GGAGCAAC AATTAATCGCATTTC-3′; and TaqMan MGB probe Bc3P with 5′ FAM label: 5′-TCACCTTGCAATGAGTGG-3′. Primers were synthesized by Sigma-Aldrich (Haverhill, UK) and the MGB probe was synthesized by Applied Biosystems (Foster City, CA). TaqMan real-time PCR was carried out using master mix consisting of 0.025 U μl^{-1} AmpliTaq Gold (Applied Biosystems) and 1 x Buffer A (supplied with AmpliTaq Gold), plus 0.2 mM each dNTP, 5.5 mM $MgCl_2$, 300 nM each primer and 100 nM TaqMan probe. DNA (1 µl) extracted from inoculated rose or pelargonium (and dilutions of these extracts), or from cultures of *B. calthae*, *B. fabae* and *B. pelargonii* was added to 24 µl of master mix, and negative controls containing nuclease-free water instead of DNA were included in each run. DNA extracts were tested in duplicate in each run, and each run was repeated at least once. Real-time PCR was carried out on an ABI Prism 7900HT instrument using the following cycling conditions: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 s and 60°C for 1 minute.

Detection by lateral flow device

LFDs for detection of *Botrytis* were purchased from Forsite Diagnostics (York, UK). Individual rose petals were placed into plastic bottles containing 5 ml Buffer C (supplied with the LFDs) and 5 stainless steel ball-bearings (5 mm diameter), and were disrupted by shaking for approximately 10 s. To test cultures of *Botrytis* species, single agar plugs (approximately 1 cm^2) were shaken with Buffer C and ball bearings for approximately 1 minute. Approximately 70 µl of the disrupted sample was applied to the release pad of each LFD and allowed to flow across the membrane. The development of a single control line indicated a negative result; a positive result was indicated by the appearance of two lines (control and test lines).

Comparison of *B. cinerea* detection methods

Detached rose petals were inoculated with approximately 200 *B. cinerea* conidia (in a volume of 4 µl) per petal and incubated for up to 55 hours, as described earlier. At 5, 29, 48 and 55 hours after inoculation two petals were removed and frozen at –80°C for subsequent DNA extraction, and a further two petals were removed and subjected to testing by LFD. The petals were examined for development of symptoms of *B. cinerea* infection at each time point, and where lesions were visible the approximate diameter was noted. DNA extractions were carried out at the end of the time course, and the resulting extracts were tested by LAMP (followed by agarose gel electrophoresis) and real-time PCR. In order to compare specificity of the methods, LFD, LAMP and real-time PCR were used to test cultures of *B. calthae*, *B. fabae* and *B. pelargonii*.

Real-time LAMP

Real-time LAMP was carried out on the Genie I instrument, using the primer concentrations given above, with 1x Isothermal MasterMix (OptiGene) containing a fluorescent intercalating dye. Reactions were held at 65°C for 20 minutes with real-time fluorescence monitoring. Real-time LAMP results were analysed in terms of Tp values (the time taken to generate a positive result). Reactions consisted of 24 µl master mix and 1 µl DNA, and negative controls containing water instead of DNA were included in each run. A tenfold dilution series of DNA extracted from *B. cinerea* in culture ranging from 65 ng μl^{-1} to 6.5 pg μl^{-1} was tested, as well as tenfold dilution series of DNA extracted from symptomatic inoculated rose petals and pelargonium leaf discs. Reactions were carried out in duplicate in each run, and each experiment was repeated at least once.

RESULTS

Optimisation and characterization of *B. cinerea* LAMP assay

In order to investigate the sensitivity of the optimised LAMP assay, a tenfold dilution series (65 ng μl^{-1} to 650 fg μl^{-1}) of DNA extracted from a culture of *B. cinerea* was tested. The lowest amount of DNA to be consistently detected was 65 pg, although 6.5 pg was amplified in some replicates (Figure 4.2), indicating that this is close to the limit of detection for the assay. The products of the *B. cinerea* assay displayed the ladder-like pattern typical of LAMP products, and amplification was not observed in the negative control reactions (Figure 4.2). Positive results were obtained for DNA extracted from

symptomatic *B. cinerea*-inoculated rose petals and pelargonium leaf discs: typical amplification products are also shown in Figure 4.2.

The optimised LAMP assay did not amplify DNA extracted from any of the non-target species that were tested, with the exception of *B. pelargonii* (data not shown).

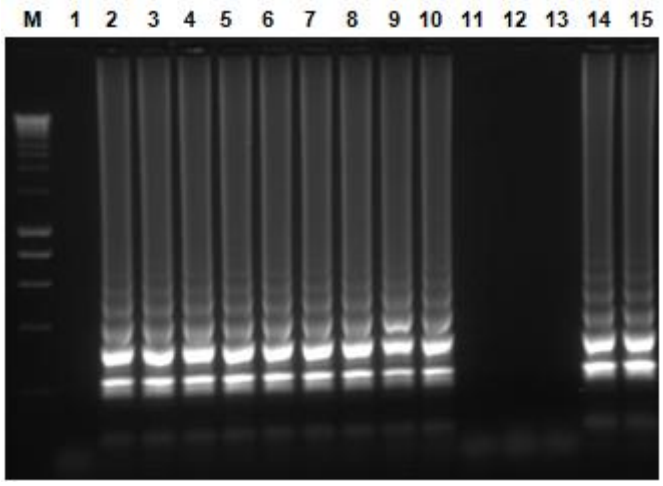

Figure 4.2. Amplification products of the *Botrytis cinerea* loop-mediated isothermal amplification assay visualized by agarose gel electrophoresis. M: marker (HyperLadder I (New England Biolabs)); lane 1: no template control; lanes 2 and 3: 65 ng *B. cinerea* DNA; lanes 4 and 5: 6.5 ng *B. cinerea* DNA; lanes 6 and 7: 650 pg *B. cinerea* DNA; lanes 8 and 9: 65 pg *B. cinerea* DNA; lanes 10 and 11: 6.5 pg *B. cinerea* DNA; lanes 12 and 13: 650 fg *B. cinerea* DNA; lane 14: DNA extracted from *B. cinerea*-infected rose; lane 15: DNA extracted from *B. cinerea*-infected pelargonium.

Comparison of *B. cinerea* detection methods

Sensitivity of the *B. cinerea* LAMP assay was compared with that of real-time PCR and *Botrytis* LFD by testing rose petals inoculated with *B. cinerea* conidia at different times after inoculation. Only real-time PCR gave a positive result 5 hours after inoculation (Table 4.1). The material remained asymptomatic at 29 hours, but *B. cinerea* was detectable by both real-time PCR and LAMP. Detection by LFD was only possible after 55 hours, at which time visible lesions of around 5 mm in diameter had developed. A similar pattern of detection using the three methods was observed when the experiment was repeated using different numbers of conidia for inoculation and correspondingly longer or shorter periods of incubation (data not shown).

Table 4.1. Detection of *B. cinerea* in inoculated rose petals at 5 to 55 hours after inoculation by TaqMan real-time PCR, LAMP followed by agarose gel electrophoresis, and lateral flow device (LFD). Detached rose petals were surface sterilized and inoculated with approximately 200 conidia per petal before being incubated at room temperature.

Time from inoculation	Real-time PCR (mean Ct value ± s.d.)*	LAMP	LFD	Symptoms (approx. lesion diameter)
5 h	+ (34.25 ± 0.56)	-	-	-
29 h	+ (29.74 ± 0.31)	+	-	-
48 h	+ (28.19 ± 0.10)	+	-	+ (1 mm)
55 h	+ (20.79 ± 0.08)	+	+	+ (5 mm)

*For duplicate reactions.

+: both replicates positive; -: both replicates negative.

The detection methods were also compared in terms of specificity for *B. cinerea* by testing closely related *Botrytis* species. As expected, the *Botrytis* LFD detected all *Botrytis* species tested (*B. calthae*, *B. fabae* and *B. pelargonii*). The LAMP assay detected *B. pelargonii*, but not *B. calthae* or *B fabae*, as described above. The TaqMan real-time PCR assay also detected *B. pelargonii*, and amplified DNA extracted from *B. calthae* with a Ct value exceeding 37.5 cycles, but did not detect *B. fabae* (data not shown).

Real-time LAMP

The tenfold dilution series of DNA extracted from a culture of *B. cinerea* was tested by real-time LAMP on the Genie I instrument, and a linear relationship was observed between DNA concentration and Tp value for concentrations between 6.5 ng μl^{-1} and 6.5 pg μl^{-1} (Figure 4.3). Tp values of 8 to 12 minutes were observed. At higher DNA concentrations the response was no longer linear, and similar Tp values were observed for 6.5 ng, 65 ng and 650 ng DNA per reaction (data not shown).

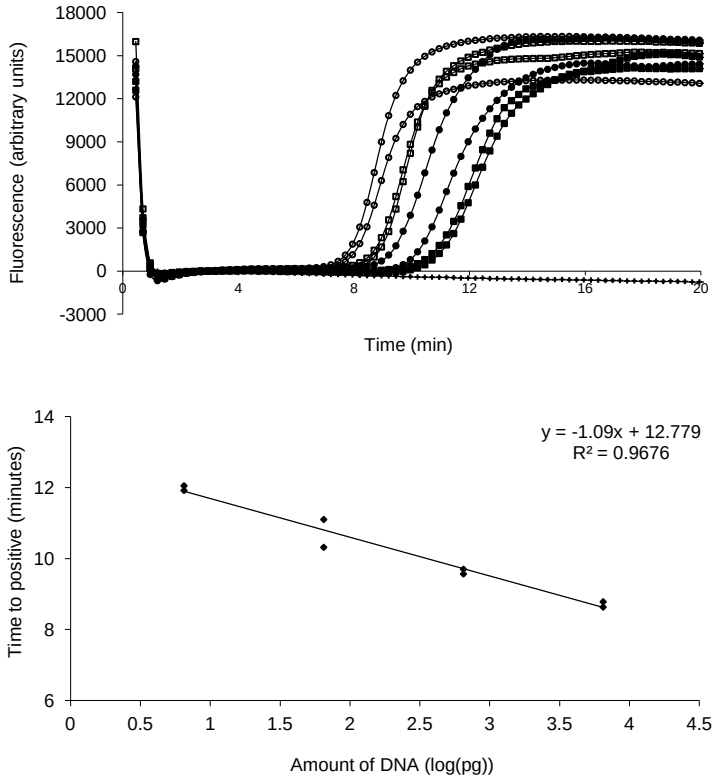

Figure 4.3. Amplification plots (top) and standard curve of time to positive result (Tp) in minutes vs amount of DNA per reaction (bottom) for a dilution series of *Botrytis cinerea* DNA tested by real-time loop-mediated isothermal amplification. Top: (○) 6.5 ng DNA, (□) 650 pg DNA, (●) 65 pg DNA, (■) 6.5 pg DNA, (+) no template control. An initial decrease in fluorescence is observed in the first 60 s as the temperature increases to the reaction temperature of 65°C.

Dilutions of DNA extracted from symptomatic *B. cinerea*-infected rose petals and pelargonium leaf discs were tested by real-time LAMP and real-time PCR, as shown in Figure 4.4. The 10^{-4} dilutions of both the rose and the pelargonium extracts were positive when tested by both methods, but the 10^{-5} dilutions were detected by real-time PCR only. The concentrations of *B. cinerea* DNA in the 10^{-5} dilutions were approximately 14 pg μl^{-1} and 3 pg μl^{-1} for rose and pelargonium, respectively, suggesting that the limit of detection when testing infected plant material was of the same order of magnitude as the limit of detection observed when testing DNA extracted from *B. cinerea* in culture (approximately 6.5 pg). The LAMP assay amplified the undiluted DNA extracts from both rose and pelargonium. In contrast, the undiluted pelargonium extract was not amplified by real-time PCR within 40 cycles, and the Ct value for the undiluted rose extract exceeded the Ct value for the 10^{-4} dilution (Figure 4.4), indicating the presence of substances inhibitory to PCR in the undiluted extracts. These results suggest that the LAMP assay has a greater tolerance of inhibitors derived from necrotic plant material than the real-time PCR assay.

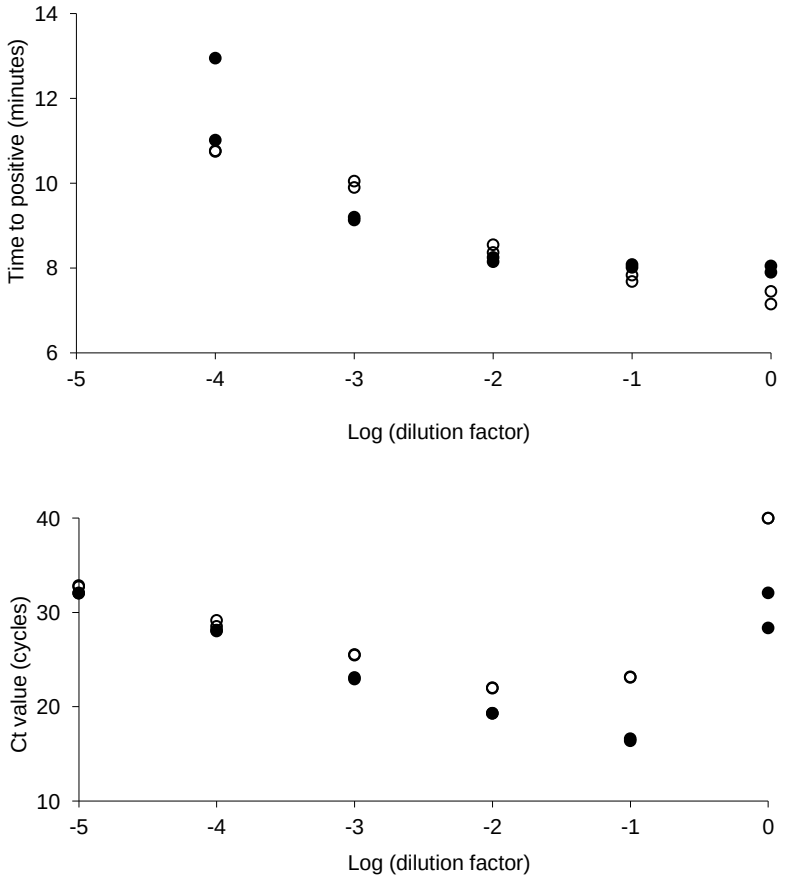

Figure 4.4. Results of real-time loop-mediated isothermal amplification (LAMP) (top) and TaqMan real-time PCR (bottom) for dilutions of DNA extracted from *B. cinerea* infected plant material: (○) rose, and (●) pelargonium. LAMP results are shown as time to positive (Tp) in minutes. For real-time PCR, a Ct value of 40 represents a negative result.

DISCUSSION

The real-time LAMP assay described in this paper was able to amplify 6.5 pg *B. cinerea* DNA in around 12 minutes (Figure 4.3). In comparison, Suarez et al. (2005) reported limits of detection of ranging from 20 fg to 20 pg for four real-time PCR assays designed to target different sequences. Mehli et al. (2005) developed a TaqMan real-time PCR assay for *B. cinerea* based on β-tubulin sequence with a limit of detection of 1 pg, and from estimates of the genome size of *B. cinerea* inferred that this assay was able to detect approximately three pathogen cells under their experimental conditions. It could therefore be estimated that the LAMP assay should be able to detect the equivalent of around 20 pathogen cells. However, rose petals inoculated with approximately 200 *B. cinerea* conidia were negative by LAMP 5 hours after inoculation, suggesting that the actual limit of detection is somewhat higher than this estimate. It remains to be determined whether this is an appropriate level of sensitivity for detection of *B. cinerea* in naturally inoculated samples of the pathogen's many hosts. However, the LAMP assay was found to be more sensitive than LFD, and detected *B. cinerea* in the early stages of infection of inoculated rose petals, prior to symptom development.

Several nucleic acid-based methods for the detection of *B. cinerea* have been described, targeting IGS (Suarez et al. 2005), β-tubulin (Brouwer et al. 2003; Mehli et al. 2005; Suarez et al. 2005; Spotts et al. 2008), cutinase A (Gachon and Saindrenan 2004), RNA helicase (Celik et al. 2009), and a SCAR marker identified by Rigotti et al. (2002) (Suarez et al. 2005; Cadle-Davidson 2008). The majority of these methods have not been screened against non-target *Botrytis* species. However, assays targeting β-tubulin and cutinase A

have been observed or predicted to cross-react with *B. fabae* (Suarez et al. 2005; Spotts et al. 2008). We tested the specificity of the *B. cinerea* LAMP assay and the IGS TaqMan assay of Suarez et al. (2005) against the three species identified by Staats et al. (2005) as those most closely related to *B. cinerea* on the basis of phylogenetic analysis of three nuclear protein-coding genes. Both assays detected the most closely related species, *B. pelargonii*, but not *B. fabae*. The TaqMan real-time PCR assay also cross-reacted weakly with *B. calthae*, but the LAMP assay was not observed to cross-react with this species. Both *B. pelargonii* and *B. calthae* are reported to have narrow host ranges, so any cross-reactivity is only potentially problematic when testing these hosts. Near species-specific monoclonal antibodies for *B. cinerea* have been described, for example the antibodies described by Bossi and Dewey (1992) which detected *B. cinerea* and *B. fabae*, but not *B. allii*. However, immunoassays developed for routine detection of *B. cinerea* have used monoclonal antibodies which detect other species in the genus *Botrytis* (Meyer et al. 2000; Dewey and Meyer 2004).

The LAMP method can be carried out without thermal cycling equipment, potentially making this method more suitable than PCR for use outside conventional laboratory facilities, where established methods are too slow, expensive or complex for routine use. This could be beneficial within industry, where rapid assessment of the levels of pathogen in crops or commodities could be used to make decisions relating to storage or processing. In order to establish the value of the LAMP assay in a particular testing scenario, it will be necessary to test naturally inoculated samples in comparison with symptom development and existing detection methods. The

sensitivity and specificity of the LAMP assay suggest that this method could be suitable for testing in situations in which existing antibody-based tests are insufficiently sensitive and where species-level specificity is required. Further to this, quantitative detection of *B. cinerea* by real-time LAMP could be particularly useful in situations where a link has been established between inoculum concentration and subsequent disease, for example, the relationship investigated by Spotts et al. (2008) between inoculum concentration and post-harvest decay of pears.

The *B. cinerea* real-time LAMP assay was observed to have a somewhat narrower dynamic range than the real-time PCR assay, but also appeared to be less affected by inhibitors when testing DNA extracted from symptomatic plant material. In previous reports, LAMP assays have shown an increased tolerance of inhibitory substances including culture media, common clinical matrices (Kaneko et al. 2007) and compounds found in soil (Tani et al. 2007c). Increased tolerance of inhibitors allows LAMP to be used in conjunction with simplified nucleic acid extraction methods (Fukuta et al. 2003b). The Food and Environment Research Agency has developed a method for extraction of nucleic acid from plant material using LFDs (Danks and Boonham 2007) which can be used in conjunction with isothermal amplification for detection of plant pathogens (Tomlinson et al. 2010c). It is likely that simplified extraction methods will typically produce DNA extracts of lower concentration than more lengthy conventional methods such as those using CTAB. However, we obtained positive results for CTAB DNA extracts diluted 1 in 10,000 (Figure 4.4), suggesting that the LAMP assay could be

sensitive enough to be used with a less efficient extraction method for some applications.

A final consideration for the deployment of LAMP for on-site detection of *B. cinerea* is the need for an internal control assay to assist in the interpretation of negative results by distinguishing true negative results from false negatives caused by inhibition or failed nucleic acid extraction. A LAMP assay based on cytochrome oxidase has been found to be suitable for use as an internal control for a range of plant species (Tomlinson et al. 2010c). Suarez et al. (2005) developed a real-time PCR internal control assay based on plant ribosomal internal transcribed spacer (ITS) sequence which was used for a number of hosts, including *Pelargonium* sp., suggesting that this could be an alternative target for development of a LAMP plant control assay. Problems may arise in attempting to detect host DNA in necrotic samples, in which case an exogenous amplification control assay could be used for the interpretation of negative results (Hartman et al. 2005).

In summary, we have designed a LAMP assay for detection of *B. cinerea* which is more sensitive than an existing LFD test and less susceptible to inhibitory substances derived from symptomatic plant material than an existing TaqMan real-time PCR assay. Use of real-time detection on the OptiGene Genie I allowed the assay to be completed in <20 minutes.

ACKNOWLEDGEMENTS

The authors wish to acknowledge funding from Forsite Diagnostics Ltd and the Proof of Concept Fund of the InterAct Partnership.

CHAPTER 5

Prepared for submission in short communication format to European Journal of Plant Pathology, 2012

J.A. Tomlinson, S. Ostoja-Starzewska, K. Webb, J. Cole, A. Barnes, M. Dickinson and N. Boonham. A loop-mediated isothermal amplification-based method for confirmation of *Guignardia citricarpa* in citrus black spot lesions.

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Data collected by J.A. Tomlinson and S. Ostoja-Starzewska.

All data analysed and manuscript prepared by J.A. Tomlinson.

A loop-mediated isothermal amplification-based method for confirmation of *Guignardia citricarpa* in citrus black spot lesions

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ABSTRACT

Guignardia citricarpa, the causal agent of citrus black spot disease, is subject to phytosanitary restrictions in the EU and USA, such that consignments of citrus are rejected at import if citrus black spot is identified on inspection. Due to the variability of black spot symptoms, positive identification solely on the basis of visual inspection is difficult, especially when lesions lack pycnidia (fruiting bodies of the anamorph *Phyllosticta citricarpa*). As an aid to visual inspection of symptoms, we have developed a method for detection of *G. citricarpa* using loop-mediated isothermal amplification (LAMP) which can be used to confirm the presence of *G. citricarpa* in black spot lesions, including those lacking pycnidia. The LAMP assay can be used to test crude extracts prepared directly from lesions on fruit, and the entire test can be completed in <40 minutes, making it faster than

previously described PCR-based methods for detection of *G. citricarpa*. The method is sufficiently simple to allow deployment of the test in the field, for example in the course of import inspections.

Keywords: Detection, citrus black spot, isothermal amplification, rapid testing

INTRODUCTION

Guignardia citricarpa Kiely (anamorph *Phyllosticta citricarpa* Van der Aa) is the causal agent of citrus black spot disease, which affects a number of economically important *Citrus* species and is subject to phytosanitary control in the EU and USA. Within the EU, citrus consignments are inspected at import, and fruit found to be infected with *G. citricarpa* is rejected. Symptoms of *G. citricarpa* infection range from typical hard spot lesions characteristic of the disease to false melanose and freckle spot, the lesions of which often lack the pycnidia required for visual confirmation of black spot (Baayen et al. 2002). Methods for isolation of the pathogen in culture or incubation of fruit to encourage formation of pycnidia are time consuming and have been found to be unreliable due to overgrowth of *G. citricarpa* by faster-growing organisms and the likelihood of false negative results (Bonants et al. 2003; Meyer et al. 2006). Most importantly, detection of *G. citricarpa* at import requires much faster methods than those based on culturing and incubation, as consignment value decreases significantly over time (Baayen et al. 2002). The current EPPO diagnostic protocol (EPPO 2009) recommends that *G. citricarpa* should be detected in symptomatic fruit by direct testing of excised lesions by PCR-based methods, without initial culturing. Several methods based on polymerase chain reaction (PCR) have been developed for detecting *G. citricarpa* in black spot lesions (Bonants et al. 2003; Meyer et al. 2006; Peres et al. 2007; van Gent-Pelzer et al. 2007). These methods allow relatively rapid detection of *G. citricarpa* in lesions with and without pycnidia, and are reported to detect *G. citricarpa* but not the common non-pathogenic endophyte *G. mangiferae* (Meyer et al. 2006; Peres et al. 2007). While real-time PCR-based detection

outside the laboratory has been demonstrated in principle for some plant pathogens (Tomlinson et al. 2005), routine PCR-based testing is generally confined to the laboratory, primarily due to the cost and complexity of thermal cycling equipment. Extremely rapid PCR reactions have been demonstrated as being technologically achievable (Wheeler et al. 2011), but most established conventional and real-time PCR methods take several hours to complete, especially when the time taken for extraction of nucleic acid is taken into account. The time taken to obtain a result can be reduced by the use of simplified extraction methods, but higher levels of inhibitory substances in crude extracts can impair the sensitivity of PCR-based methods and sensitivity can be reduced (Kaneko et al. 2007). We have developed a method for specific detection of *G. citricarpa* in lesions excised from infected citrus using loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000; Nagamine et al. 2001; Nagamine et al. 2002). LAMP does not require thermal cycling, reaction times are short (often less than 30 min), and tolerance of substances which can inhibit PCR allows crude extracts to be tested without impaired sensitivity (Kaneko et al. 2007). We have developed a LAMP assay for detection of *G. citricarpa* which can be performed using the field-portable Genie II instrument (OptiGene, Horsham, UK) in a format that is suitable for deployment at import inspection. Following initial characterisation of the LAMP assay in comparison with real-time PCR, the assay was used with a crude extraction method for detection of *G. citricarpa* in different lesion types, with a total test duration of <40 minutes.

MATERIALS AND METHODS

LAMP primers for *G. citricarpa* were designed in the internal transcribed spacer (ITS) region of the rDNA gene. Primer design was based primarily on alignments of ITS sequences of *G. citricarpa* (Accession numbers FJ538311, FJ769680, FJ769681), *G. mangiferae* (FJ769748) and the recently described pathogen of pomelo *Phyllosticta citriasiana* (FJ538363, FJ538364) (Wulandari et al. 2009), in combination with BLAST analysis of potential target regions. Samples were also tested for host DNA using LAMP primers targeting plant cytochrome oxidase sequence, as previously described (Tomlinson et al. 2010c). Primer sequences are shown in Table 5.1.

Table 5.1. Primers and probes used in this study.

Primer name	Sequence (5′-3′)	Assay (source)
Gc F3	GGTTTTGACCCGGGCGG	*G. citricarpa* LAMP (this study)
Gc B3	CGATGATTCACTGAATTCTGCAA	
Gc FIP	AATAATCGCTGGAGTTTTGTATACTGGCGC CCMCAGYCTAGTCTC	
Gc BIP	CTGTGTAGTCCTGAGAATTCATTTAATGTT TCGCTGCGTTCTTCATCG	
Gc F-loop	CCAGGCGTCCTGGCCTA	
Gc B-loop	AATAAAACTTTCAACAACGGATCTC	
COX F3	TATGGGAGCCGTTTTTGC	Plant cytochrome oxidase (COX) LAMP (Tomlinson et al. 2010c)
COX B3	AACTGCTAAGRGCATTCC	
COX FIP	ATGGATTTGRCCTAAAGTTTCAGGGCAGG ATTTCACTATTGGGT	
COX BIP	TGCATTTCTTAGGGCTTTCGGATCCRGCGT AAGCATCTG	
COX F-Loop	ATGTCCGACCAAAGATTTTACC	
COX B-Loop	GTATGCCACGTCGCATTCC	
GcF1	GGTGATGGAAGGGAGGCCT	*G. citricarpa* TaqMan real-time PCR (van Gent-Pelzer et al. 2007)
GcR1	GCAACATGGTAGATACACAAGGGT	
GcP1	AAAAAGCCGCCCGACCTACCTTCA[1]	
COX F	CGTCGCATTCCAGATTATCCA	COX TaqMan real-time PCR (based on Weller et al. 2000)
COX RW	CAACTACGGATATATAAGRRCCRRAACTG	
COX probe	AGGGCATTCCATCCAGCGTAAGCA[2]	

[1]FAM (6-carboxyfluorescein) reporter (5′); TAMRA (tetra-methylcarboxyrhodamine) quencher (3′).

[2]JOE (6-carboxy-4,5-dichloro-2,7-dimethoxy fluorescein) reporter (5′); BHQ1 (Biosearch Technologies, Novato, CA) quencher (3′).

DNA extracted from cultures was used to characterise the sensitivity and specificity of the LAMP assay. Cultures of *G. mangiferae* (CBS123374, CBS115053) and *P. citriasiana* (CBS123393, CBS120485) obtained from the Centraalbureau voor Schimmelcultures (Utrecht, Netherlands) and *G. citricarpa* (Fera ref 11848) were used for assay characterisation. DNA was extracted from mycelium scraped from the surface of cultures grown on cornmeal agar (*Guignardia* isolates) or oatmeal agar (*P. citriasiana* isolates), and DNA was extracted using the CTAB-based method described by Suarez et al. (2005) with the modifications that only one chloroform extraction step was performed and the DNA was resuspended in 100 µl nuclease-free water. Approximate DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, USA) or a Qubit fluorometer and dsDNA HS Assay kit (Life Technologies, Carlsbad, USA). DNA extracts were diluted in nuclease-free water as required and stored at -20°C prior to testing by real-time LAMP and real-time PCR.

In order to further compare the performance of the LAMP assay with that of a previously published TaqMan real-time PCR assay (van Gent-Pelzer et al. 2007), 24 samples of citrus fruit intercepted by the UK Plant Health and Seeds Inspectorate were tested for *G. citricarpa* using both methods. All samples displayed symptoms of disease, some of which were suspected to be black spot, but lesions containing pycnidia were observed on one sample only. For comparative testing of intercepted citrus samples, DNA was extracted using a semi-automated CTAB-based extraction method as follows. Sections of peel (up to 2 cm^2 total sample size) were excised from the surface of the fruit, placed in heavy-gauge polythene bags (Bioreba, Reinach, Switzerland)

and briefly snap frozen in liquid nitrogen. A wooden mallet was used to manually disrupt the frozen material, then 2-3 ml CTAB buffer (2% CTAB; 120 mM sodium phosphate pH 8; 1.5 M NaCl; 2% Antifoam B) was added to the bag, and the sample was ground using a HOMEX 6 homogeniser (Bioreba). The ground sample (1.5 ml) was transferred to a 2 ml microcentrifuge tube and centrifuged at approximately 10,000 x g for 5 minutes. The supernatant (700 µl) was mixed with 200 µl chloroform by vortexing, then centrifuged at approximately 13,000 x g for 5 minutes. The aqueous layer (500 µl) was mixed with 500 µl isopropanol and 50 µl Magnesil Paramagnetic Particles (MPPs) and incubated at room temperature for 10 minutes. DNA was extracted using a KingFisher mL instrument to transfer the MPPs sequentially through 1 ml GITC Buffer (5.25 M guanidine thiocyanate; 50 mM Tris-HCl pH 6.4, 20 mM EDTA, 1.3% (w/v) Triton X-100) and two washes in 1 ml 70% ethanol, followed by elution in 200 µl 1 x TE buffer. DNA was stored at -20°C prior to testing by real-time LAMP and real-time PCR.

In order to investigate the use of a crude DNA extraction method, hard spot and freckle spot lesions were identified on *G. citricarpa*-infected fruit. Each lesion was excised using a sterile scalpel (sample size approximately 2-4 mm diameter) and placed into a small heavy-gauge plastic bag with 600 µl Buffer C (Forsite Diagnostics, York, UK). The material was manually disrupted using a small hammer, then the sample was transferred to a centrifuge tube. Crude extracts prepared in this way were added directly to real-time LAMP reactions. In total, 16 hard spot lesions, six freckle spot lesions and two samples each consisting of four freckle spot lesions pooled together were tested by real-time LAMP. In addition, eight samples taken from

the rind of non-infected fruit were tested as negative controls. Crude extracts were observed to be less stable during prolonged storage at 4°C than DNA extracts stored at 4°C or -20°C, so crude extracts were tested in a single run, with at least two replicate reactions per sample in each run.

LAMP for *G. citricarpa* or COX was carried out in 25 µl reactions consisting of 15 µl Isothermal Master Mix (OptiGene), 2 µM each internal primer (FIP and BIP), 200 nM each external primer (F3 and B3), 1 µM each loop primer (F-loop and B-loop), and 1 µl DNA or crude extract. Reactions were incubated at 65°C for 30 minutes followed by a slow annealing step of (0.05°C s^{-1}) from 95°C to 75°C with fluorescence monitoring to determine the annealing temperature of the amplification products. The Isothermal Master Mix contains a fluorescent dye for real-time detection. Initial testing was carried out on an ABI 7500 (Life Technologies), in which case ROX passive reference dye (Life Technologies) was added at 0.0625 µl per 25 µl reaction; subsequent testing was carried out using a Genie II instrument (OptiGene), and reactions were run without ROX. Results were interpreted in terms of Tp (time to positive) values and amplification product annealing temperatures. During assay development, amplification products were also analyzed by gel electrophoresis using 1.2% agarose gels containing ethidium bromide at 0.5 µg ml^{-1}. Reactions were carried out in duplicate, and reactions containing water instead of DNA were included in each run as negative controls. All runs were performed at least twice, except as otherwise stated. TaqMan real-time PCR was carried out on an ABI 7900HT instrument (Life Technologies) using the primers and probe for detection of *G. citricarpa* developed by van Gent-Pelzer et al. (2007) and primers and probe for detection of the plant cytochrome

oxidase gene (COX) based on those developed by Weller et al. (2000). Primer and probe sequences are shown in Table 5.1. Each 25 µl reaction consisted of 1 x Buffer A, 0.625 units AmpliTaq Gold (Life Technologies), 200 µM each dNTP, 5.5 mM $MgCl_2$, 300 nM each primer, 100 nM probe and 1 µl DNA. The following cycling conditions were used: 10 minutes at 95°C followed by 40 cycles of 15 s at 95°C and 1 minute at 60°C. Reactions were performed in duplicate and reactions containing water instead of DNA were included in each run as negative controls. Results were interpreted in terms of Ct (cycle threshold) values.

RESULTS AND DISCUSSION

Figure 5.1 shows LAMP results for detection of *G. citricarpa*. The limit of detection was determined to be between 60 fg and 600 fg DNA; all replicate reactions containing 600 fg DNA were observed to be positive, while at 60 fg DNA some replicates were negative in some runs, indicating that the limit of detection lies between these values. This is comparable to the limit of detection for the real-time PCR assay, which we observed to be approximately 60 fg (data not shown), and which van Gent-Pelzer et al. (2007) reported as 10 fg (this difference is likely to be attributable to inaccuracies in quantification of DNA). The amplification products of the LAMP assay had the ladder-like appearance typical of LAMP products when visualised by agarose gel electrophoresis (Figure 5.1). For all positive reactions, the annealing temperature of the amplification product was observed to be in the range 86.1 – 87.0°C (data not shown). No amplification was observed for extracts from *G. mangiferae* or non-infected citrus. Amplification was observed for higher

concentrations of DNA (30 pg per reaction or above) extracted from cultures of *P. citriasiana* (Figure 5.2); however, the amplification products of these reactions had annealing temperatures in the range 89.1 – 89.7°C (with a second peak in the range 83.3 – 84.2°C), which could easily be distinguished from the amplification products of *G. citricarpa* DNA. The products were also distinguishable when visualised by agarose gel electrophoresis (Figure 5.2).

Table 5.2 shows a summary of the results of testing 24 intercepted citrus samples for *G. citricarpa* by real-time LAMP and real-time PCR. Eleven samples tested positive for *G. citricarpa* by both methods, and 13 tested negative by both methods. DNA quality and yield for all extracts was confirmed by COX real-time PCR and COX real-time LAMP.

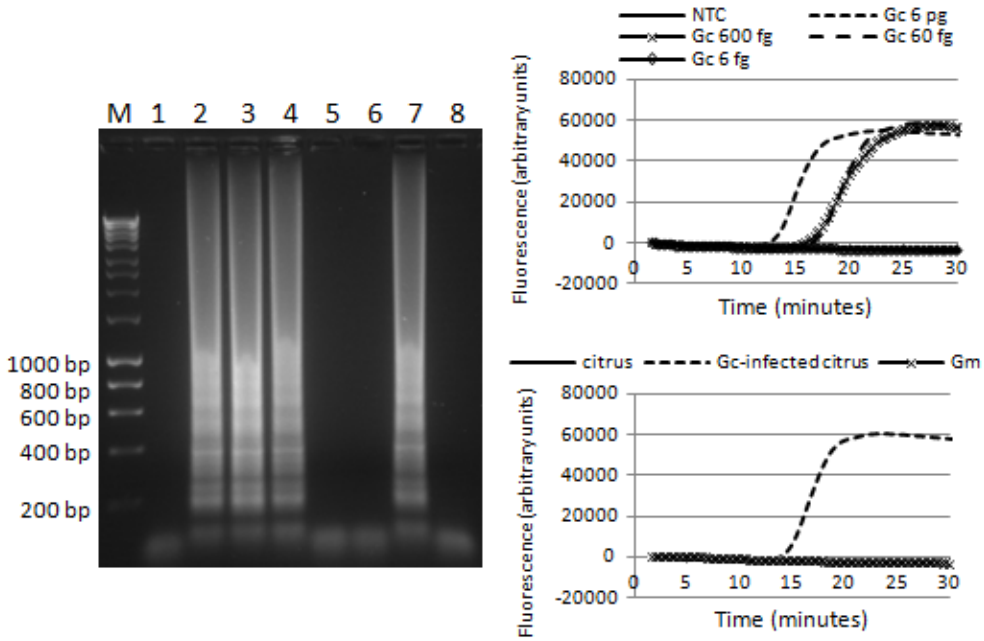

Figure 5.1. Detection of *Guignardia citricarpa* by loop-mediated isothermal amplification (LAMP). Left: visualisation of amplification products by agarose gel electrophoresis. M: HyperLadder I marker (Bioline, London, UK); lane 1: no template control; lanes 2-5: tenfold dilutions of *G. citricarpa* DNA (lane 2: 6 pg; lane 3: 600 fg; lane 4: 60 fg; lane 5: 6 fg); lane 6: DNA from non-infected citrus; lane 7: DNA from *G. citricarpa*-infected citrus; lane 8: *G. mangiferae* DNA (approximately 270 pg). Right: amplification plots of real-time LAMP. NTC = no template control; Gc = *G. citricarpa* DNA; Gm = *G. mangiferae* DNA (approximately 270 pg).

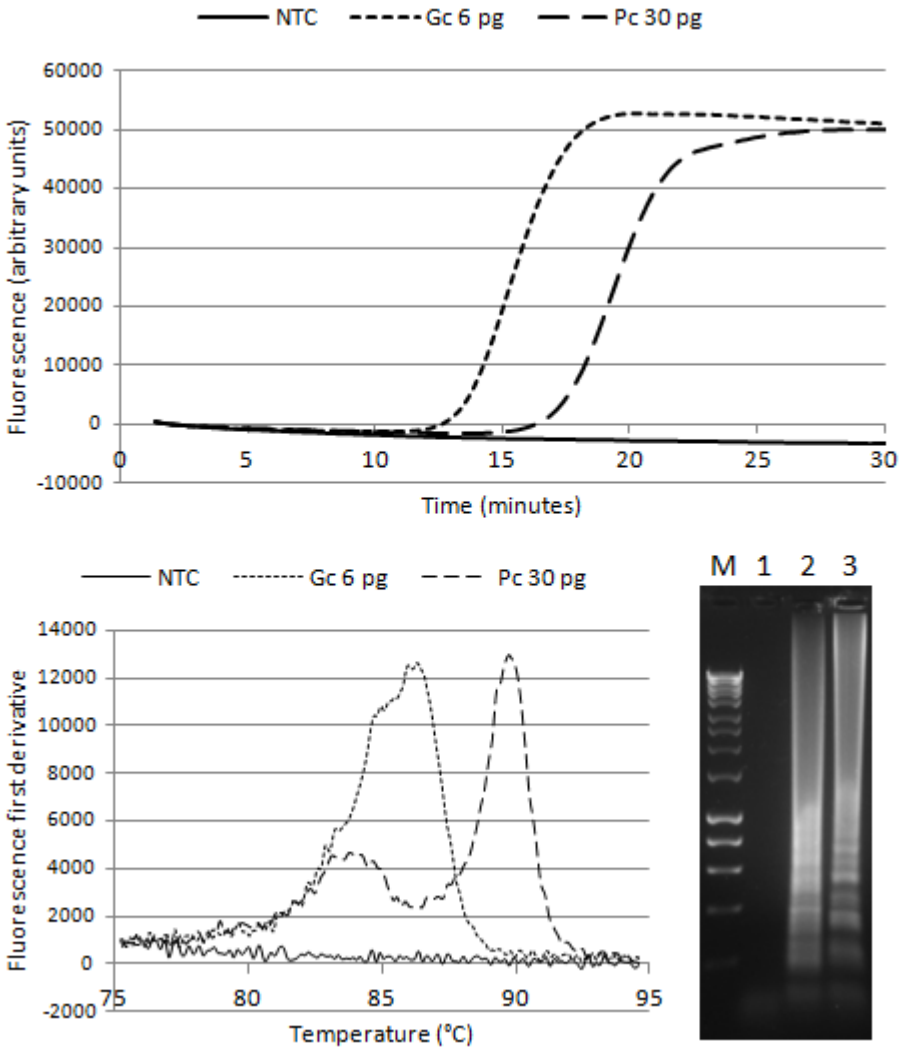

Figure 5.2. Loop-mediated isothermal amplification (LAMP) for DNA extracted from *Guignardia citricarpa* and *Phyllosticta citriasiana*. Top: amplification plots of real-time LAMP. Bottom left: annealing temperature analysis of LAMP products. NTC = no template control; Gc = *G. citricarpa* DNA; Pc = *P. citriasiana* DNA. Bottom right: visualisation of amplification products by agarose gel electrophoresis. M: marker (HyperLadder I); lane 1: no template control; lane 2: 6 pg *G. citricarpa* DNA; lane 3: 30 pg *P. citriasiana* DNA.

Table 5.2. Results of comparative testing of 24 intercepted citrus samples for *Guignardia citricarpa* and host plant DNA (cytochrome oxidase, COX) by real-time loop-mediated isothermal amplification (LAMP) and TaqMan real-time PCR.

Sample number	Host	*G. citricarpa* LAMP	COX LAMP	*G. citricarpa* TaqMan	COX TaqMan
1	*Citrus sinensis*	+	+	+	+
2	*C. sinensis*	+	+	+	+
3	*C. sinensis*	+	+	+	+
4	*C. limon*	+	+	+	+
5	*Citrus* sp.	+	+	+	+
6	*C. sinensis*	+	+	+	+
7	*C. macroptera*	+	+	+	+
8	*C. macroptera*	+	+	+	+
9	*C. macroptera*	+	+	+	+
10	*Citrus* sp.	+	+	+	+
11	*C. sinensis*	+	+	+	+
12	*C. sinensis*	-	+	-	+
13	*C. reticulata*	-	+	-	+
14	*C. macroptera*	-	+	-	+
15	*C. limon*	-	+	-	+
16	*C. aurantifolia*	-	+	-	+
17	*Citrus* sp.	-	+	-	+
18	*C. aurantifolia*	-	+	-	+
19	*C. limon*	-	+	-	+
20	*C. aurantifolia*	-	+	-	+
21	*C. limon*	-	+	-	+
22	*Citrus* sp.	-	+	-	+
23	*C. aurantifolia*	-	+	-	+
24	*C. aurantifolia*	-	+	-	+

+ = positive result; - = negative result.

Typical LAMP results for testing crude extracts from lesions excised from citrus peel are shown in Figure 5.3. Amplification was observed within 20 minutes for both classes of lesion that were tested, with annealing/melt temperatures in the range 86.0 – 86.8°C. All freckle spot lesions tested positive in all replicate reactions. Of the hard spot lesions tested, one out of 16 was positive in one of the duplicate reactions, and one was negative in both reactions. Two reactions gave annealing peaks outside the expected range, but in both cases the other reaction gave an annealing peak at the expected temperature. Using the *G. citricarpa* LAMP primers, no amplification was observed for samples taken from the peel of non-infected fruit; all *G. citricarpa*-negative samples gave positive reactions with the COX assay, with Tp values of approximately 12 minutes and product annealing temperatures in the range 84.9 – 85.1°C (data not shown).

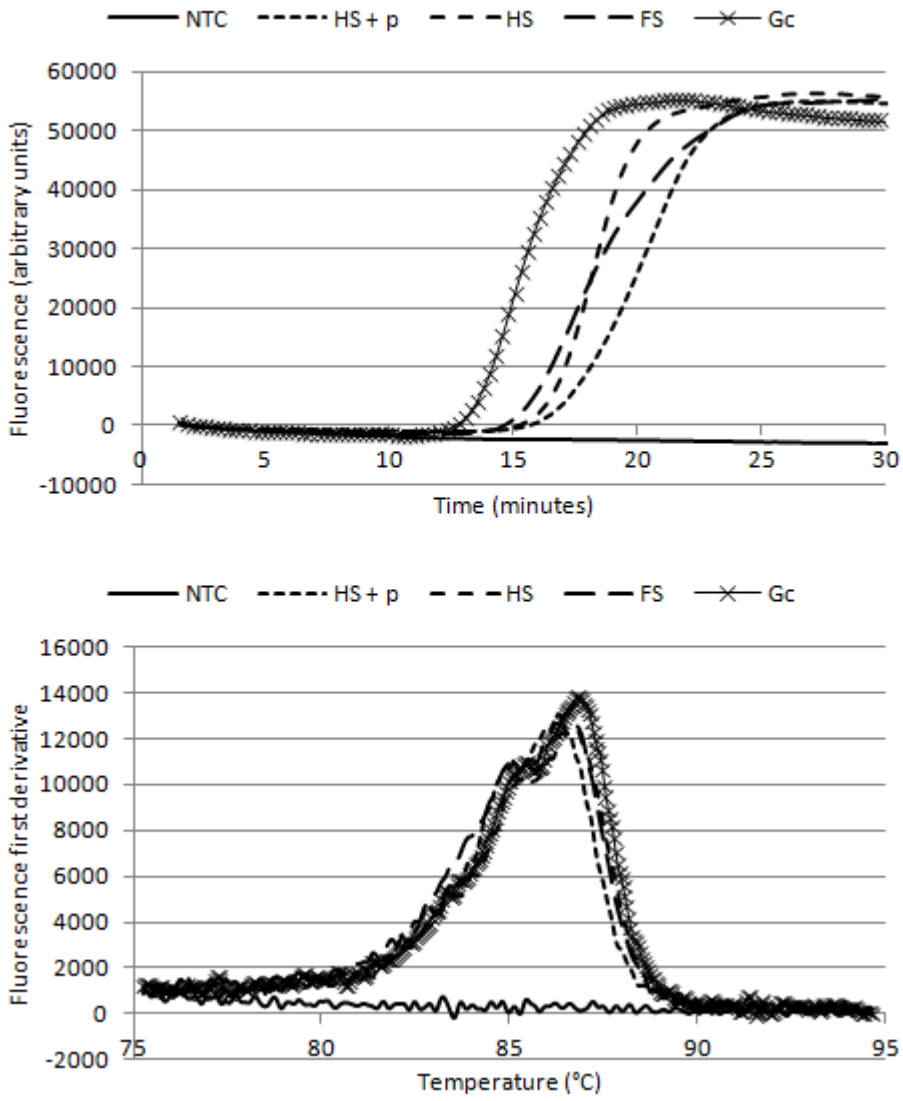

Figure 5.3. Detection of *Guignardia citricarpa* in crude extracts from citrus black spot lesions by real-time loop-mediated isothermal amplification (LAMP). Top: amplification plots of real-time LAMP. Bottom: annealing temperature analysis of LAMP products. NTC = no template control; HS + p = hard spot lesion with pycnidia; HS = hard spot lesion without pycnidia; FS = freckle spot lesion without pycnidia; Gc = *G. citricarpa* DNA (6 pg).

The observed limit of detection of the *G. citricarpa* LAMP assay was therefore found to be sufficient for detection of the pathogen in crude extracts prepared from lesions excised from symptomatic fruit. The ability to test crude extracts is a critical factor affecting the feasibility of deploying this method at import inspection. The test can be completed in less than 40 minutes, including crude extract preparation and annealing temperature analysis, and very few manipulations are required.

The majority of previous papers reporting PCR-based diagnosis of *G. citricarpa* have emphasised the need to discriminate between *G. citricarpa* and the ubiquitous endophyte *G. mangiferae* due to the difficulty of reliably discriminating these organisms solely on the basis of morphological and growth characteristics in culture. A greater challenge for DNA-based testing is the discrimination of *G. citricarpa* from more closely related species such as the recently described pathogen *Phyllosticta citriasiana* (EPPO 2009; Wulandari et al. 2009). While DNA extracted from *P. citriasiana* in culture was amplified by the *G. citricarpa* LAMP primers, the amplification product was clearly distinguished on the basis of its annealing temperature. The taxonomy of the genus *Phyllosticta* is currently being resolved (Glienke et al. 2011; Wikee et al. 2011; Wang et al. 2012) and further testing will be required to determine how the LAMP assay, as well as the PCR-based assays on which the EPPO protocol is based, will react with newly emerging pathogenic and endophytic *Phyllosticta* spp. associated with citrus.

ACKNOWLEDGEMENTS

Funding for this work was provided by Defra. The authors would like to thank Jim Beckley of Fera Plant Health and Seeds Inspectorate for providing helpful feedback during development of these methods.

CHAPTER 6

Accepted for publication in Journal of Virological Methods, 2012

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TaqMan real-time PCR assays provided by I.P. Adams.

Testing performed by J.A. Tomlinson and S. Ostoja-Starzewska.

Data analysed and manuscript prepared by J.A. Tomlinson.

Loop-mediated isothermal amplification for rapid detection of the causal agents of cassava brown streak disease

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ABSTRACT

The causal agents of cassava brown streak disease have recently been identified as *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV). Primers have been developed for rapid detection of these viruses by reverse transcription loop-mediated isothermal amplification (RT-LAMP). Performance of the RT-LAMP assays compared favourably with

published RT-PCR and real-time RT-PCR methods. Furthermore, amplification by RT-LAMP is completed in 40 minutes and does not require thermal cycling equipment. Modification of the RT-LAMP reactions to use labelled primers allowed rapid detection of amplification products using lateral flow devices containing antibodies specific to the incorporated labels, avoiding the need for fluorescence detection or gel electrophoresis.

Keywords: diagnosis; loop-mediated isothermal amplification; cassava brown streak disease; CBSV; UCBSV

INTRODUCTION

Cassava brown streak disease (CBSD), caused by *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) (genus *Ipomovirus*, family *Potyviridae*), causes yield losses and reduced marketability (i.e. reduced economic yield) of cassava roots (Hillocks et al. 2001) in areas of East Africa, including Kenya, Tanzania and Uganda. Development of necrosis in infected roots prior to harvesting undermines the value of cassava as a food security crop in areas prone to drought. The disease was first described in 1936 (Storey, 1936; Hillocks and Jennings, 2003), and CBSV was identified as the causal agent in 2001 (Monger et al. 2001b). A variant of CBSV identified initially in samples from the highland regions of Uganda subsequently was determined to be sufficiently genetically distinct to be described as a separate species, *Ugandan cassava brown streak virus* (UCBSV) (Mbanzibwa et al. 2009b, Monger et al. 2010, Winter et al. 2010). UCBSV is now widespread in Kenya, Tanzania and Uganda and was recently reported in Burundi (Bigirimana et al. 2011), with further credible reports in Rwanda and Democratic Republic of Congo (Legg et al. 2011). The potential threat of CBSVs (CBSV and UCBSV) to the Great Lakes region of East Africa has been highlighted by the Food and Agriculture Organization (FAO 2011).

Symptoms of CBSD can resemble those resulting from other causes (for example, senescence) and expression is inconsistent, so reliable detection based on symptoms alone is not possible. Nucleic acid-based methods allow detection of CBSV and UCBSV in material without symptoms, and small sequence differences can be exploited to allow differentiation of closely related taxa such as the two causal species of CBSD. A number of conventional RT-

PCR assays have been developed for detection of CBSV and UCBSV (Monger et al. 2001a; Mbanzibwa et al. 2011). The primer set developed by Monger et al. (2001a) pre-dates the description of UCBSV as a separate species and targets sequence specific to CBSV. The primers developed by Mbanzibwa et al. (2011) can be used to detect both species in a two-step RT-PCR in which the amplification products are discriminated on the basis of amplicon length determined by agarose gel electrophoresis. More recently, one-step real-time RT-PCR assays for the detection of CBSV and UCBSV have been developed (Adams et al. 2012). ELISA-based diagnostic systems for CBSV are commercially available for which little or no performance data has been published.

Nucleic acid-based approaches to detection of plant pests and diseases can have advantages of sensitivity and specificity over antibody-based methods, such as ELISA (Ward et al. 2004; Mumford et al. 2006; Boonham et al. 2008; Smith et al. 2008). In addition to sensitivity and specificity, other considerations are taken into account in the selection of detection methods for different applications. Developing new target-specific antibodies can be time consuming and costly, but nucleic acid-based methods have greater flexibility in design for new targets, or emergent strains of existing targets (Le et al. 2010). Conversely, once developed, antibodies can be incorporated into lateral flow devices suitable for field testing in non-laboratory situations. Scales of analysis and cost are also important. Abarshi et al. (2010) discussed RT-PCR and RNA extraction methods for detection of CBSVs in terms of the cost of reagents and plasticware per sample. Additional factors to be taken into account include the costs of labour, equipment and infrastructure, and the time

taken to obtain results using different methods. Levels of technical skill in operation and how this is reflected in salary are other variables. It should be noted that costs are situation-specific, and the costs of operating diagnostic laboratories will be different in developed and developing countries.

Isothermal amplification methods have been developed which have the potential to overcome some of the cost barriers limiting uptake of PCR-based testing while exceeding the sensitivity and/or specificity of ELISA-based methods. Loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000; Nagamine et al. 2001) is a method for specific amplification of target nucleic acid sequences without thermal cycling, the use of which has been demonstrated for detection of various plant pathogens (Tomlinson and Boonham 2008). LAMP primers are designed to generate amplification products containing single-stranded loop regions to which primers can bind without denaturation. LAMP reactions can be extremely rapid and highly efficient, and the amount of amplification product generated can allow the use of novel detection methods. For example, colour change methods enable non-instrumented detection (Iwamoto et al. 2003; Goto et al. 2009). An alternative approach is the use of lateral flow devices (LFDs) to detect labels incorporated into the amplification products, allowing multiple products to be discriminated without gel electrophoresis (Kiatpathomchai et al. 2008; Tomlinson et al. 2010c). Flexibility of detection formats and short reaction times (typically one hour or less) make LAMP potentially amenable to incorporation into simplified workflows suitable for use in non-laboratory settings or in laboratories with limited facilities.

This article describes the development of RT-LAMP primers for detection and discrimination of CBSV and UCBSV. LAMP assays were optimised and characterised using the Genie II instrument (OptiGene, Horsham, UK) for real-time LAMP. Performance of the LAMP assays was compared to that of the conventional RT-PCR assay of Mbanzibwa et al. (2011) and the real-time TaqMan RT-PCR assays of Adams et al. (2012) in parallel testing of naturally infected field samples. In addition, a rapid method for detection of LAMP products using lateral flow devices was demonstrated for simultaneous detection of the amplification products for CBSV, UCBSV and a plant internal control gene (cytochrome oxidase).

MATERIALS AND METHODS

Plant material and RNA extraction

Initial development of the LAMP assays was carried out using RNA extracted from leaves of *Nicotiana benthemiana* infected with CBSV or UCBSV. Further testing was carried out using RNA extracted from cassava leaves which had been collected in the field in the Mwanza province of Tanzania, where both CBSV and UCBSV have been detected. The samples of leaf material were dried on-site before being sent to the laboratory in the UK for subsequent testing (Adams et al. 2012).

RNA was extracted from fresh or dried leaf material using a modified CTAB extraction method. Samples of approximately 0.15 g (fresh) or 0.05 g (dried) material were homogenised in liquid nitrogen in a mortar and pestle before the addition of 2 ml CTAB lysis buffer (2% CTAB; 100 mM Tris-HCl, pH 8.0; 20 mM EDTA; 1.4 M NaCl; 1% sodium sulphite; 2% PVP). The

homogenate was incubated at 65°C for 15 minutes, an equal volume of chloroform:isoamyl alcohol (24:1) was added, and the sample was centrifuged for 10 minutes at approximately 12,000 x g. The aqueous layer (800 µl) was transferred to a new tube with an equal volume of 4 M LiCl and incubated overnight at 4°C. The samples were centrifuged for 25 minutes at 13,500 x g then the pelleted RNA was resuspended in 50 µl nuclease-free water and subjected to clean-up using the RNeasy extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. RNA was eluted in a total volume of 100 µl nuclease-free water and was stored at -80°C prior to testing. For sensitivity testing, RNA was diluted in nuclease-free water to produce a tenfold dilution series. For testing naturally infected field samples, total RNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, MA, USA) prior to testing by two-step RT-PCR; RT-LAMP and TaqMan RT-PCR were performed using 1 µl undiluted RNA extracts.

RT-LAMP

LAMP primers were designed based on alignments of published CBSV and UCBSV coat protein sequences (Abarshi et al. 2010; Mbanzibwa et al. 2009a, Monger et al. 2010, Winter et al. 2010). Alignments were constructed using the Clustal V method of the MegAlign program (DNAStar, WI, USA). GenBank accession numbers of the sequences used in the alignments were as follows: CBSV: FJ821794, FN423416, FN423417, FN423418, FN434436, FN434437, GQ329864; UCBSV: FJ039520, FJ185044, FN433930, FN433931, FN433932, FN433933, FN434109, NC_012698. BLAST analysis indicated that the regions selected for assay design lacked homology between

CBSV/UCBSV and non-target species, including other Potyviruses. Primers were designed to optimise discrimination between CBSV and UCBSV. Degenerate bases were incorporated into primers to mitigate against intraspecific variation where necessary. Primer sequences are shown in Table 6.1. An assay for detection of plant cytochrome oxidase (COX) was also used as a control assay (Tomlinson et al. 2010c). Real-time RT-LAMP was carried out on a Genie II instrument (OptiGene) in 25 µl reactions containing 15 µl Isothermal Master Mix (OptiGene), 200 nM each external primer (F3 and B3), 2 µM each internal primer (FIP and BIP), 1 µM each loop primer (F-Loop and B-Loop), 1.2 units ThermoScript reverse transcriptase (Life Technologies, CA, USA) and 1 µl RNA. Reactions were incubated at 50°C for 10 minutes, then 65°C for 30 minutes with fluorescence monitoring: the Isothermal Master Mix contains a fluorescent double-stranded DNA binding dye. To measure the annealing/melting temperature of the amplification products, the reactions were subjected to a slow annealing step (0.05°C s^{-1}) from 95°C to 75°C with fluorescence monitoring. Reactions containing water instead of RNA were included in each run as negative controls. Results were interpreted in terms of Tp (time to positive) values. During assay development and initial characterization, amplification products were further analysed by electrophoresis using 1.2% agarose gels containing ethidium bromide at a final concentration of 0.5 µg ml^{-1}. All RT-LAMP runs were performed at least twice.

Table 6.1. Loop-mediated isothermal amplification primers.

Primer	Sequence (5′-3′)
CBSV F3	CGACRATGAGGAAAATAATGAGAAAT
CBSV B3	GAACAACTTRGTTTTATTTCTACCAA
CBSV FIP	TTTTCAATGCTTGTATACCCAGCACGATCAGAAT AGTGTGWCTGCTGGA
CBSV BIP	GGTATTGACTTCCTAGCCGAAGCATTAGCAGCCA GTATTTGATGTTT
CBSV F-Loop[1]	TTCGGGCTGCTTTTATYACAA
CBSV B-Loop[2]	ACAAYTGTCACAAAGCCAACT
UCBSV F3	AATYCCAACWARTGCTCTTGAGAT
UCBSV B3	TATTAACTCCATATGCTTTAGCAAC
UCBSV FIP	CCTTTGAGAGCGYGGAATCAAGACDTTCAAGCCT CCAAA
UCBSV BIP	TTTCCTGGCATAYRTACCTCCATTGCCCAATTYTC AACTTCAA
UCBSV F-Loop[3]	CAAATGTAAGCTGACTGTGAYAC
UCBSV B-Loop[2]	CTCAYGCTATAGATAAYCAACTTGC
COX F3	TATGGGAGCCGTTTTTGC
COX B3	AACTGCTAAGRGCATTCC
COX FIP	ATGGATTTGRCCTAAAGTTTCAGGGCAGGATTTC ACTATTGGGT
COX BIP	TGCATTTCTTAGGGCTTTCGGATCCRGCGTAAGC ATCTG
COX F-Loop[4]	ATGTCCGACCAAAGATTTTACC
COX B-Loop[2]	GTATGCCACGTCGCATTCC

[1]5′-labelled with fluorescein isothiocyanate (FITC) when used with LFD detection of LAMP products.

[2]5′-labelled with biotin when used with LFD detection of LAMP products.

[3]5′-labelled with digoxigenin (DIG) when used with LFD detection of LAMP products.

[4]5′-labelled with Texas Red when used with LFD detection of LAMP products.

TaqMan real-time RT-PCR

Testing by TaqMan real-time RT-PCR was carried out using the primers and probes described by Adams et al. (2012) as shown in Table 6.2. Real-time RT-PCR was carried out on an ABI 7900HT instrument using 25 µl reactions containing 1 x Buffer A, 0.625 units AmpliTaq Gold (Life Technologies, CA, USA), 10 units Revertaid reverse transcriptase (Fermentas, St. Leon-Rot, Germany), 0.2 mM each dNTP, 5.5 mM $MgCl_2$, 300 nM each primer, 100 nM probe and 1 µl RNA. Cycling conditions were 30 minutes at 48°C and 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Reactions containing water instead of RNA were included in each run as negative controls. Results were interpreted in terms of Ct (cycle threshold) values. All reactions were performed in duplicate in each run.

Conventional RT-PCR

Two-step RT-PCR was carried out as described by Mbanzibwa et al. (2011) using the primers shown in Table 6.2. Briefly, reverse transcription was carried out in 25 µl reactions containing approximately 2 µg total RNA (up to a volume of 10 µl), 1 x M-MLV Reaction Buffer (Promega, WI, USA), 0.5 mM each dNTP, 1.4 µM not1 dT primer (AACTGGAAGAATTGGCGGCCGCAGGAA$(T)_{18}$), and 200 units M-MLV reverse transcriptase (Promega). A mixture of RNA and primer was heated to 70°C for 5 minutes then placed on ice, after which the remaining components were added and the reactions incubated at 42°C for 60 minutes. For samples where the total RNA concentration was less than 200 ng μl^{-1}, 10 µl undiluted RNA extract was used for reverse transcription. PCR was carried out in 25 µl

reactions containing 4 µl of the reverse transcription reaction, 1 x Taq Buffer (Fermentas), 2.5 mM $MgCl_2$, 0.28 mM each dNTP, 400 nM primer CBSDDR, 400 nM primer CBSDDF2 and 1.5 units Taq polymerase (Fermentas), with thermal cycling conditions of 2 minutes at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 51°C and 30 s at 72°C. Amplification products were analysed by agarose gel electrophoresis as described above. All reactions were performed at least twice, and reactions containing water instead of RNA or cDNA were included in all reverse transcription and PCR runs, respectively.

Table 6.2. RT-PCR primers and TaqMan real-time RT-PCR primers and probes.

Primer	Sequence (5′-3′)	Reference
CBSDDR	GGATATGGAGAAAGRKCTCC	Mbanzibwa et al. 2011
CBSDDF2	GCTMGAAATGCYGGRTAYACAA	Mbanzibwa et al. 2011
UCBSV forward	GATYAARAAGACITTCAAGCCTCC AAA	Adams et al. 2012
UCBSV reverse	AATTACATCAGGRGTTAGRTTRTCC CTT	Adams et al. 2012
UCBSV probe[1]	TCAGCTTACATTTGGATTCCACGCT CTCA	Adams et al. 2012
CBSV forward	GCCAACTARAACTCGAAGTCCATT	Adams et al. 2012
CBSV reverse	TTCAGTTGTTTAAGCAGTTCGTTCA	Adams et al. 2012
CBSV probe[2]	AGTCAAGGAGGCTTCGTGCYCCTC	Adams et al. 2012

[1]Probe labelled with FAM (6-carboxyfluorescein) reporter (5′) and TAMRA (tetra-methylcarboxyrhodamine) quencher (3′).

[2]Probe labelled with VIC (Life Technologies) reporter (5′) and TAMRA quencher (3′).

Lateral flow device detection of RT-LAMP products

LAMP was carried out using labelled primers in the combinations shown in Table 6.1. For these experiments, LAMP reactions were incubated in a heated block instead of the Genie II instrument. For each assay, one loop primer (B-loop) was labelled with biotin, and the other loop primer (F-loop) was labelled with either FITC, DIG or Texas Red, as indicated in Table 6.1. LAMP using labelled primers resulted in amplification products labelled with two ligands, allowing the products to be detected by LFD. PCRD-4 devices containing reagents to bind to FITC, DIG and Texas Red on the membrane and latex functionalised to bind to biotin were obtained from Forsite Diagnostics (York, UK). Each sample was amplified using each of the three RT-LAMP primer sets (CBSV, UCBSV and COX), then the reactions were combined, diluted and applied to a single device. The labelled LAMP reactions were diluted in LFD Dilution Buffer (Forsite Diagnostics): 1 µl DIG-labelled product, 2 µl FITC-labelled product and 2 µl Texas Red-labelled product were added to 1 ml Dilution Buffer and inverted to mix. Approximately 70 µl of the diluted combined reactions was applied to the release pad of the device. Devices were left to develop for at least 15 minutes before being examined. The presence of detectable levels of each target was indicated by the presence of a line at the corresponding position of the device as follows: position 1: DIG-labelled product (UCBSV); position 2: not used; position 3: FITC-labelled product (CBSV); position 4: Texas Red-labelled product (COX).

RESULTS

RT-LAMP assays for detection of CBSV and UCBSV

Both CBSV and UCBSV were detected by real-time RT-LAMP using a single incubation temperature of 65°C for 40 minutes (data not shown). Initial testing indicated that sensitivity was consistently enhanced by the use of an initial 10-minute incubation at 50°C followed by 65°C for 30 minutes, and all further testing was carried out using these conditions. Figure 6.1 shows typical results for detection of CBSV, UCBSV and plant cytochrome oxidase using the corresponding RT-LAMP assays. Each assay was specific for its target species, and amplification was typically observed within 10 minutes at 65°C; amplification using the plant control COX assay was typically observed within 10-15 minutes. Amplification products visualised by agarose gel electrophoresis had the ladder-like appearance typical of LAMP products (Notomi et al. 2000), as shown in Figure 6.1. Annealing temperatures in the ranges 83-84°C were observed for the amplification products of the CBSV and UCBSV assays; annealing temperatures in the range 84-85°C were observed for amplification products of the COX assay (data not shown).

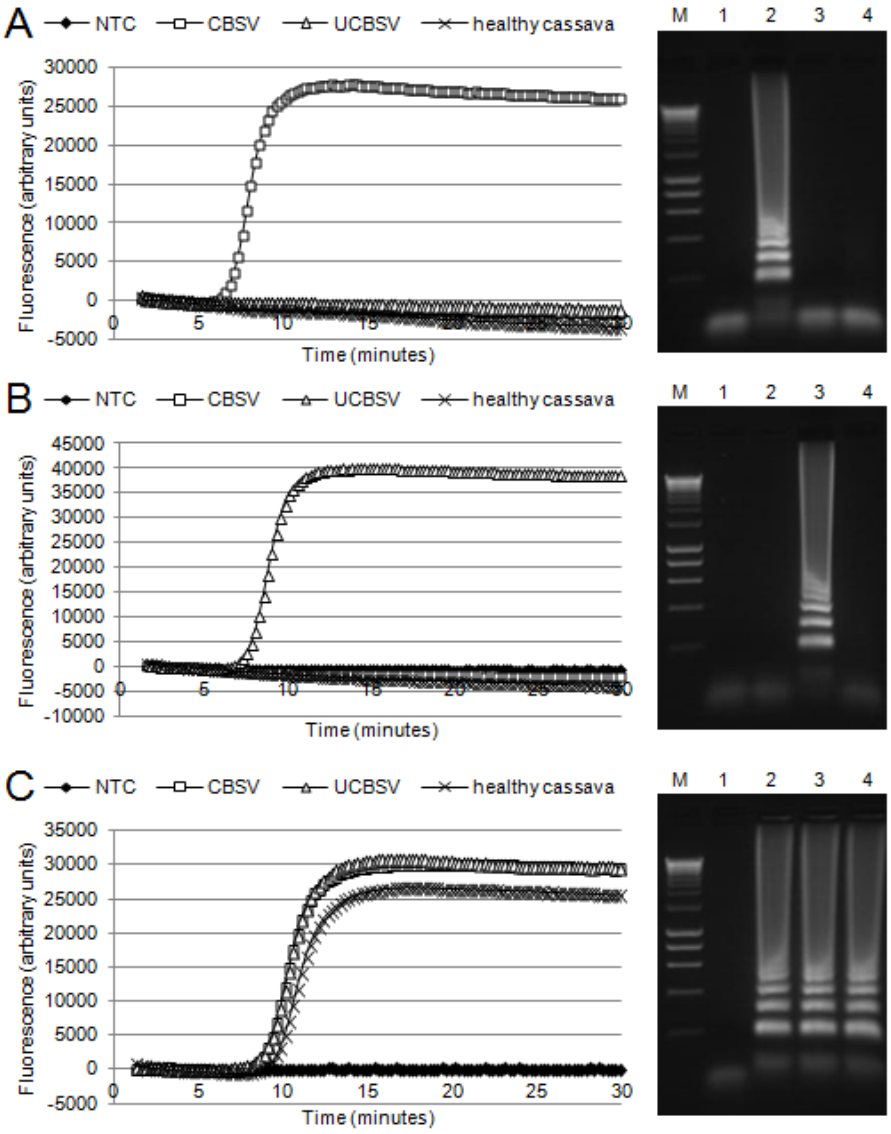

Figure 6.1. Loop-mediated isothermal amplification for detection of CBSV and UCBSV. Amplification plots (left) and agarose gel electrophoresis of amplification products (right) for CBSV-infected and UCBSV-infected indicator plants (*Nicotianae benthemiana*) and non-infected cassava tested by LAMP for CBSV (A), UCBSV (B) and plant cytochrome oxidase (C). NTC: no-template control. M: marker (HyperLadder I, New England Biolabs); lane 1: NTC; lane 2: CBSV-infected plant; lane 3: UCBSV-infected plant; lane 4: healthy cassava.

RT-LAMP assay sensitivity

Figure 6.2 shows the results of real-time RT-LAMP for dilution series of RNA extracted from CBSV- and UCBSV-infected plants, in comparison with the results of TaqMan RT-PCR for the same dilution series. Similar limits of detection were observed for RT-LAMP and TaqMan RT-PCR, and results of replicated reactions were consistent at concentrations exceeding the limit of detection. The limit of detection of the RT-LAMP assays was observed to be at dilution factors of 10^{-2}-10^{-3}, and at these dilutions amplification was not observed in all replicates (Figure 6.2).

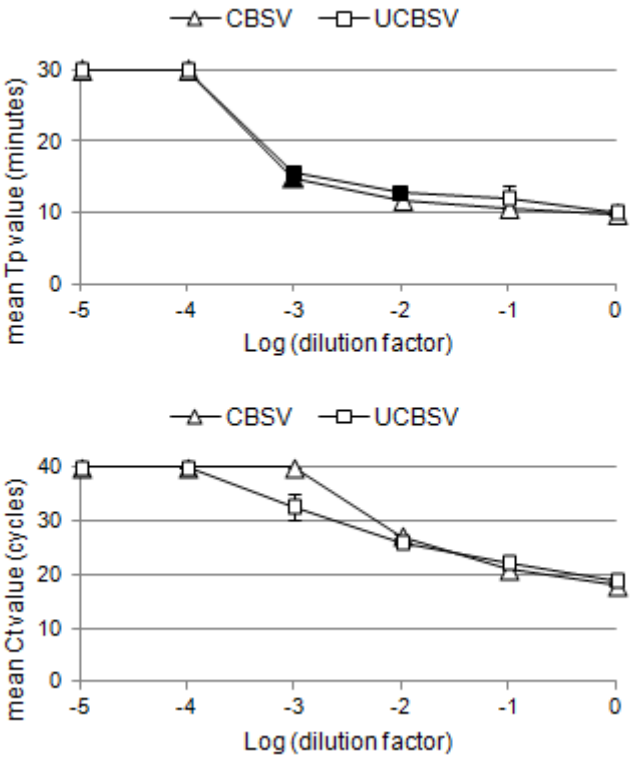

Figure 6.2. Results of real-time RT-LAMP (top) and TaqMan real-time RT-PCR (bottom) for CBSV and UCBSV for dilutions of RNA extracted from infected plant material. Real-time RT-LAMP results are shown as time to positive (Tp) values; TaqMan RT-PCR results are shown as Ct values. Negative real-time RT-LAMP and TaqMan RT-PCR reactions were assigned values of 30 minutes and 40 cycles, respectively. Filled symbols indicate real-time RT-LAMP reactions for which one replicate was negative and the other positive: only the Tp value for the positive replicate is shown. All other results shown are mean values for duplicate reactions; error bars show standard deviations.

Comparison of detection methods

Thirty dried and stored field samples were tested by real-time RT-LAMP, two-step RT-PCR and TaqMan RT-PCR: comparative results are shown in Table 6.3. The two-step RT-PCR method of Mbanzibwa et al. (2011) specifies the amount (2 µg) of total RNA to be used in the reverse transcription reaction, whereas the RT-LAMP and TaqMan RT-PCR methods used a defined volume (1 µl) of undiluted RNA per reaction. For the RNA extracts from dried and stored cassava leaves used in this study, the total RNA yield ranged from 2.9 ng μl^{-1} to 850.7 ng μl^{-1}, with the majority of extracts (87%) containing in excess of 50 ng μl^{-1}. A maximum volume of 10 µl RNA could be accommodated by the reverse transcription reaction, and no additional concentration of RNA extracts was performed, so the volume of RNA extract used for reverse transcription varied from 2.4 to 10 µl, corresponding to 28.6 ng - 2 µg per reaction.

Twenty three samples tested positive for UCBSV by TaqMan RT-PCR, of which six were also positive for CBSV. TaqMan RT-PCR Ct values ranged from 19.7 to 30.3 and 15.3 to 21.0 for UCBSV and CBSV, respectively. The results for real-time RT-LAMP were in agreement with those for TaqMan RT-PCR, with the exception of one sample (B54) that was negative by RT-LAMP but positive for UCBSV by TaqMan RT-PCR. This sample gave the highest TaqMan RT-PCR Ct value (mean 30.3 cycles), indicating the lowest UCBSV titre of all the UCBSV-positive samples, and also contained the lowest concentration of total RNA (2.9 ng μl^{-1}), indicating that RNA extraction was less efficient for this sample. Real-time RT-LAMP Tp values ranged from 8 minutes 30 seconds to 19 minutes 15 seconds, and 9 minutes 15 seconds to 15

minutes 15 seconds for UCBSV and CBSV, respectively. Four samples (including sample B54) were positive by TaqMan RT-PCR but negative by conventional RT-PCR, and for a further three samples only one out of two replicate reactions was positive by RT-PCR, suggesting that these samples contained levels of virus that were close to the limit of detection of the RT-PCR assay. All samples that were negative by TaqMan RT-PCR were also negative by RT-LAMP and RT-PCR. Negative RT-LAMP results for CBSV and UCBSV were confirmed by testing with the plant control (COX) LAMP assay: Tp values in the range 10 – 12 minutes were observed for all samples (data not shown).

Table 6.3. Comparison of detection of CBSV and UCBSV by LAMP, RT-PCR and TaqMan real-time RT-PCR for naturally infected field samples.

	UCBSV			CBSV		
	TaqMan	PCR	LAMP	TaqMan	PCR	LAMP
B3	+	+/-	+	-	-	-
B7	-	-	-	-	-	-
B13	-	-	-	-	-	-
B15	+	+	+	-	-	-
B20	+	+/-	+	-	-	-
B22	+	+	+	+	+	+
B24	+	-	+	+	+/-	+
B29	+	+	+	+	+	+
B30	+	-	+	+	+	+
B34	+	-	+	+	+	+
B40	+	+	+	-	-	-
B43	+	+	+	-	-	-
B44	+	+	+	-	-	-
B46	+	+	+	-	-	-
B47	+	+	+	+	+	+
B48	+	+	+	-	-	-
B49	+	+	+	-	-	-
B50	+	+	+	-	-	-
B52	+	+	+	-	-	-
B54	+	-	-	-	-	-
B55	+	+	+	-	-	-
B58	+	+	+	-	-	-
B59	+	+	+	-	-	-
B60	+	+	+	-	-	-
B61	+	+	+	-	-	-
CT4	-	-	-	-	-	-
CT5	-	-	-	-	-	-
CT7	-	-	-	-	-	-
CT8	-	-	-	-	-	-
CT11	-	-	-	-	-	-

+/-: one out of two replicate reactions positive; for all other results, all replicates gave concurrent results.

Lateral flow device detection of RT-LAMP products

Figure 6.3 shows typical results for detection of CBSV and UCBSV using labelled RT-LAMP followed by LFD detection. It was possible to combine the CBSV, UCBSV and COX LAMP assays in a multiplex reaction containing all 18 LAMP primers; however, this resulted in reduced sensitivity of detection of the target present in the lowest concentration when more than one target was amplified (data not shown). Since mixed infections are common and it is necessary to distinguish accurately between single and mixed infections, reactions were carried out in simplex and combined prior to detection by LFD. Amplification products were detected by the presence of lines at positions 1 (DIG: UCBSV), 3 (FITC: CBSV) and 4 (Texas Red: COX plant control).

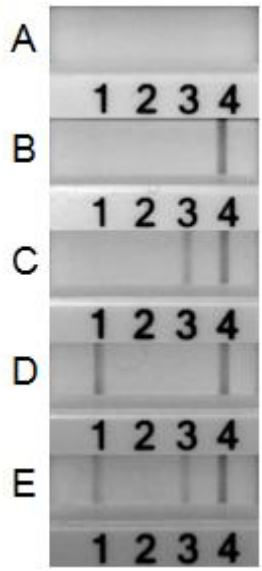

Figure 6.3. Detection of labelled LAMP products using lateral flow devices. Labelled loop primers were used to generate amplification products containing two labels: biotin was used in all three assays; the second label was FITC, DIG or Texas Red for the CBSV, UCBSV and plant control COX assays, respectively. Results are shown for A: no template control; B: healthy cassava; C: CBSV-infected plant; D: UCBSV-infected plant; E: dual-infected plant. DIG- FITC-, and Texas Red-labelled products are indicated by the presence of lines at positions 1, 3 and 4, respectively (line 2 was not used).

DISCUSSION

With the emergence of CBSD, the development and use of methods for detection of CBSVs has been an objective of the Great Lakes Cassava Initiative (GLCI). Implementation of the TaqMan RT-PCR assays developed by Adams et al. (2012) by GLCI partners in Uganda, Kenya, Tanzania, Burundi, Rwanda and DR Congo has shown that there are major differences across the region in terms of capacity and know-how, and challenges exist in technical support, maintenance of equipment and sourcing of reagents (issues of particular relevance to plant health and disease diagnosis in Africa have been discussed previously by Smith et al. (2008). In this context, there may be significant advantages in the use of LAMP assays with non-instrumented LFD detection, or with relatively low-cost instruments such as the Genie II. Abarshi et al. (2010) discussed in detail the prohibitive costs of CBSV detection by RT-PCR using kits for RNA extraction and RT-PCR. The sensitivity of RT-LAMP is equivalent to or better than that of two-step RT-PCR, the per-test consumable costs are similar, and the equipment costs for LAMP carried out using a water bath or heated block are considerably lower. In addition, RT-LAMP is significantly faster and requires fewer manipulations than two-step RT-PCR, enabling throughput to be increased without requiring additional resource. As an indication, RT-LAMP with LFD detection could be completed in less than 1 hour, TaqMan RT-PCR in approximately 2 hours, and 2-step RT-PCR in approximately 2.5 to 3 hours plus the time required for gel electrophoresis. For these reasons, RT-LAMP appears to be a promising alternative to RT-PCR for testing for CBSV and UCBSV.

The RT-LAMP assays described here enabled detection of CBSV and UCBSV strains in samples found to be with or without virus by TaqMan RT-PCR using assays for which the specificity and limit of detection have been established (Adams et al. 2012). The single anomalous result in comparative testing of 30 naturally infected field samples was recorded for the sample which gave the highest Ct value by TaqMan RT-PCR. The TaqMan RT-PCR result indicated that this sample contained the lowest UCBSV titre of the positive samples tested, and the false negative RT-LAMP result is probably attributable to this being below the threshold of detection of the RT-LAMP assay. More false negative results were observed for two-step RT-PCR in comparison with TaqMan RT-PCR and RT-LAMP. Abarshi et al. (2010) reported a higher sensitivity for one-step RT-PCR in comparison with two-step RT-PCR (using a different primer set), but concluded that a two-step protocol is preferable for deployment due to the lower cost. The false negative results recorded here for conventional RT-PCR are likely to reflect the lower sensitivity of this assay in comparison with TaqMan RT-PCR.

The majority of previously published methods for PCR-based detection of CBSVs have used CTAB-based methods for RNA extraction. Such methods are considered to be suitable due to the low cost relative to kit-based methods (Abarshi et al. 2010) and the ability to scale the method up or down for different sample sizes without significantly increasing the cost. Several previous reports have described the use of LAMP and RT-LAMP in conjunction with simplified nucleic acid extraction methods (Le et al. 2010; Tomlinson et al. 2010a; Hodgetts et al. 2011), which could further facilitate the rapid use of these methods in non-laboratory or low-resource settings. Such

methods may compromise sensitivity of detection, which could have a knock-on effect on how the tests are deployed, for example, for confirmatory testing of samples displaying clear symptoms. However, maximising the simplicity of nucleic acid-based testing – comprising nucleic acid extraction, amplification and detection – would allow these methods to be deployed in situations where testing is currently impossible or extremely limited. An advantage of the high sensitivity of real-time PCR is the ability to test bulked samples. Adams et al. (2012) demonstrated the ability to detect 1% infection in a field with 95% probability by testing 300 leaves in pools of 10 leaves per sample. Further testing is required to establish equivalent detection limits for RT-LAMP, but the sensitivity of the CBSV and UCBSV RT-LAMP assays approaches that of the TaqMan RT-PCR assays, suggesting that it may be possible to adopt a similar sampling strategy. However, the testing of bulked samples may not be compatible with the use of simplified RNA extraction methods.

The CBSV and UCBSV RT-LAMP assays were developed using an instrument for real-time monitoring of fluorescence; however, alternative detection methods may be more accessible in laboratories where testing using conventional PCR and gel electrophoresis is already established but real-time PCR is not accessible. An alternative to electrophoresis is the detection of labels incorporated into amplification products (Kiatpathomchai et al. 2008; Tomlinson et al. 2010c) using label-specific antibodies in a lateral flow device format. This method is substantially faster than electrophoresis and requires no equipment, and furthermore the devices can be used generically for any LAMP assays labelled in the same way. The per-test cost is higher for LFD detection than electrophoresis, but the ability to detect multiple differently-labelled

amplification products on a single device partially offsets this. Non-homogeneous detection methods such as electrophoresis and LFDs require the reaction tubes to be opened after amplification, so extreme care must be taken to avoid contamination of equipment and reagents with amplification products. This is best achieved by rigorously separating all pre- and post-amplification manipulations. Steps to avoid carry-over contamination should be taken for all amplification methods, but this is especially true for LAMP which generates very large amounts of amplification product. However, LAMP-based methods comprise relatively few manipulations, so it is possible to effectively control against contamination even with minimal facilities. Furthermore, the use of disposable LFDs in the post-amplification detection step can be helpful in minimising the risk of carry-over contamination.

ACKNOWLEDGMENTS

This work was supported through a sub-grant with Catholic Relief Services as the grantee of the Bill and Melinda Gates Foundation award for the Great Lakes Cassava Initiative.

CHAPTER 7

Paper in preparation

J.A. Tomlinson, M.J. Dickinson, and N. Boonham. Loop-mediated isothermal amplification and alternately-binding quenching probe technology for quantitative detection of plant pathogens.

Author contribution:

Project supervised by M. Dickinson and N. Boonham.

Data collected and analysed and manuscript prepared by J.A. Tomlinson.

Loop-mediated isothermal amplification and alternately-binding quenching probe technology for quantitative detection of plant pathogens

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ABSTRACT

Established methods for quantitative detection of plant pathogens using PCR or isothermal alternatives to PCR are typically based on real-time monitoring of amplification. Instrumentation for real-time monitoring is relatively complex, however, and it is often necessary to run samples in parallel with a set of standards. In many cases, such as statutory testing for quarantine plant pathogens, on-site detection is not required to be quantitative. Other applications, however, require methods which are at least semi-quantitative, in addition to the requirement that the methods should be rapid, simple and relatively inexpensive. A potentially simple approach to semi-quantitative detection is the use of alternately-binding quenching (AB-Q) probes with PCR or isothermal amplification. In order to investigate this approach, a LAMP assay with AB-Q probe was developed for the fungal plant pathogen *Botrytis cinerea*. Preliminary results are presented which suggest that

this method has the potential to be used for semi-quantitative detection of pathogens *in planta* using simple instrumentation. However, assay development and optimisation are more time consuming than for assays using LAMP in more conventional formats, due to the requirement to construct suitable competitor oligonucleotides, and further testing will be required to fully evaluate the performance of this method.

INTRODUCTION

On-site detection of plant pathogens can be achieved using isothermal amplification methods, which can be performed more rapidly than PCR-based methods that require thermal cycling. Loop-mediated isothermal amplification (LAMP) is a method for specific detection of nucleic acid targets, which combines speed and simplicity with levels of sensitivity and specificity approaching those of PCR, making this a suitable method for on-site use. Many applications require only qualitative results indicating the presence or absence of the target organism, but some applications require quantitative information about the amount of target in a sample, for example, where pathogen or inoculum levels are used to inform disease management actions or decisions within the production chain. Quantitative results may be expressed as a specific value (such as a numbers of cells or colony forming units, or an amount of nucleic acid) or may be semi-quantitative, for example, categorising pathogen levels as high, medium or low.

LAMP can be used with simple end-point detection methods using colour changes or lateral flow devices (Tomlinson et al. 2010c), but these methods generate qualitative results. Quantitative LAMP can be achieved by real-time monitoring of amplification, using either fluorescence detection (Mekata et al. 2009; Tomlinson et al. 2010b) with an instrument such as the Genie II (OptiGene, UK) or measurement of turbidity (Mori et al. 2004). Quantitative real-time methods often require standards to be run in parallel with the samples. This is achievable in the laboratory using high-throughput instruments, for example, for real-time PCR. However, instruments suitable for field use typically have a much lower capacity (typically 8 or 16 samples), and

the running of standards uses up a substantial proportion of the available reactions. The ideal method for quantitative detection of plant pathogens outside the laboratory would use simple equipment and avoid the need to run standards, while maintaining performance characteristics such as sensitivity, specificity and robustness.

Tani et al. (2007c) described a method for quantification of a specific DNA target using LAMP with a competitor oligonucleotide and a fluorescently-labelled alternately-binding probe (referred to as an AB probe or AB-Q probe), and a similar approach has been used with PCR for quantification without construction of a standard curve (Tani et al. 2007a, b). In the competitive LAMP / AB-Q probe approach, the ratio of target DNA to a known concentration of competitor DNA can be determined by measurement of fluorescence from the AB-Q probe. Due to the specific design of the probe and the competitor, the probe's fluorescence is quenched when bound to the target, but not if it is bound to the competitor. Kurata et al. (2001) describe the use of oligonucleotide probes labelled with BODIPY-FL which exhibit significant quenching of fluorescence when hybridised to complementary DNA containing guanines at and adjacent to the position of a fluorescently labelled cytosine at the probe's 5′ end (Figure 7.1). A competitor molecule can be designed in which the guanine bases are replaced with non-quenching cytosine bases, but which is otherwise identical to the target sequence. The target and the competitor are amplified with equal efficiency by the same primers, as they differ by a minimal number of bases and have equal G/C content, so the overall ratio of target and competitor molecules is maintained over the course of amplification. LAMP is highly efficient, generating a large

amount of amplification product, such that at the end of amplification all probe molecules are bound to amplified DNA. At this point, the fluorescence signal will be quenched to a degree that is dependent on the proportion of probe molecules that are bound to amplification product of the target compared to the proportion that are bound to the amplification product of the competitor. As a result, the amount of target present in the sample can be inferred from the degree of quenching, by comparing fluorescence before and after amplification.

Competitive amplification methods (based on PCR or LAMP) have the advantage that quantification should not be affected by inhibitors carried over from the sample matrix, since inhibition will affect amplification of the target and the competitor equally and the ratio of the two amplification products will not change. Combined with the observation that LAMP assays can be more tolerant of inhibitors than PCR-based methods, this approach could facilitate accurate quantification of targets in challenging matrices, such as soil. Tani et al. (2007c) reported that in the presence of substances including humic acid, real-time LAMP was less susceptible to inhibition than real-time PCR, but also that target DNA was more accurately quantified by LAMP using an AB-Q probe than by real-time LAMP. The objective of this work was therefore to develop a LAMP assay with AB-Q probe for *in planta* detection of *Botrytis cinerea*, and to perform a preliminary assessment of the potential advantages and disadvantages of this method.

MATERIALS AND METHODS

DNA extraction

DNA was extracted from cultures of *B. cinerea* and *B. cinerea*-inoculated rose petals and pelargonium leaf discs as described by Tomlinson et al. 2010b (see Chapter 4).

LAMP primers and AB-Q probe design

The LAMP assay with AB-Q probe was based on a previously developed LAMP assay for *Botrytis cinerea* (Tomlinson et al. 2010b) which targets the intergenic spacer region (IGS) of the nuclear ribosomal DNA (rDNA). An AB-Q probe was designed such that the base at the 5′ end of the probe is a BODIPY-FL-labelled cytosine, and the probe binds to target sequence containing two additional guanines immediately adjacent to the probe binding site (Figure 7.1). A phosphate modification at the 3′ end prevents extension of the AB-Q probe by DNA polymerase. The AB-Q probe was located at the position of the reverse loop primer (B-loop) in the original LAMP assay (this primer was omitted from AB-Q LAMP reactions). All oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany); primer sequences are shown in Figure 4.1 (Chapter 4).

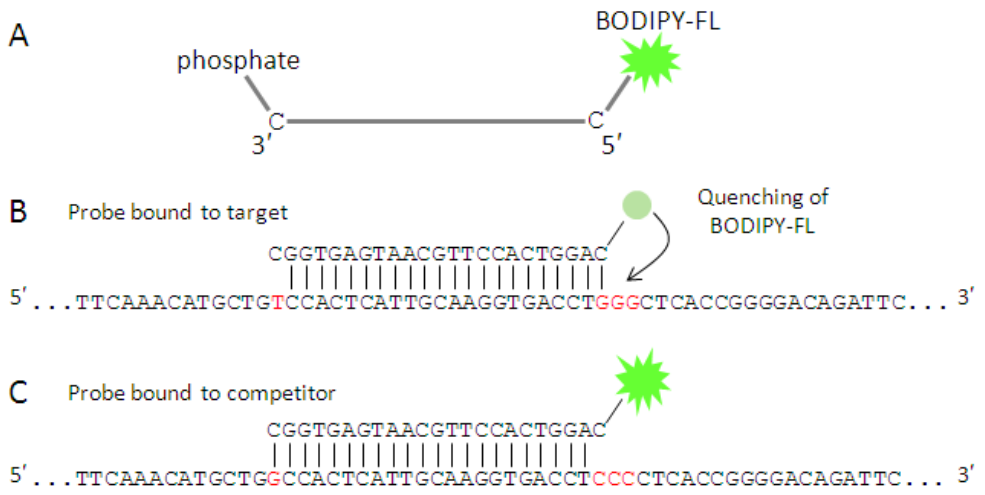

Figure 7.1. AB-Q probe design. **A**: The AB-Q probe incorporates BODIPY-FL at the 5′ end and a phosphate modification at the 3′ end to prevent extension by DNA polymerase. **B**: When bound to the target sequence, BODIPY-FL fluorescence is quenched by proximity to guanine bases in the target DNA. **C**: When bound to the competitor sequence, BODIPY-FL is not quenched, as the guanine bases have been replaces by cytosine bases. Base differences between the target and competitor are shown in red. The 3′ base of the probe is mismatched when bound to the target but matched when bound to the competitor to compensate for the match / mismatch at the 5′ base.

Competitor design and construction

The competitor was designed to differ from the target DNA as shown in Figure 7.1. In summary, the guanine corresponding to the 5′ end of the AB-Q probe and the two adjacent guanines were changed to cytosines, such that BODIPY-FL fluorescence would be quenched when the probe was bound to the target DNA but not the competitor. In addition, a thymine corresponding to a cytosine at the 3′ end of the AB-Q probe (resulting in a C/T mismatch between the AB-Q probe and the target DNA) was changed to a guanine, to compensate for the mismatch at the 5′ end (see Figure 7.1). The complete competitor consisted of a 635 bp region of *B. cinerea* IGS sequence incorporating the region targeted by the LAMP assay, with the modification of four base pairs as described above.

The competitor was constructed by overlap extension PCR based on the method described by Zentilin and Giacca (2007), using the primers shown in Table 7.1 and *B. cinerea* DNA as the template. PCR was carried out using primer pairs ext F2 / mod R1 and mod F1 / ext R2, respectively, to produce two amplification products which overlap by 17 bases. Primers mod F1 and mod R1 were designed to introduce the sequence differences required in the competitor. PCR was carried out in 50 µl reactions containing 1 x GoTaq Flexi PCR buffer (Promega, WI, USA), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1.25 units GoTaq, 1 µM each primer and 2 µl *B. cinerea* DNA (or water for no-template controls). PCR cycling conditions were 95°C for 2 minutes followed by 35 three-step cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 45 s, followed by a final extension step of 72°C for 5 minutes. PCR products were run on a 1.2% agarose gel containing 1x GelRed dye (Biotium, CA, USA) with

Hyperladder I (Bioline, UK), and the approximate product sizes were confirmed (ext R2 / mod F1: 331 bp; mod F1 / ext R2: 321 bp). The two amplification products were transferred from the gel using a band stab procedure into a reaction containing 1 x GoTaq Flexi PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 1.25 units GoTaq polymerase, heated to 95°C for 2 minutes, subjected to a slow annealing step from 95°C to 50°C at approximately 4.5°C s^{-1}, held at 50°C for 2 minutes, and finally incubated at 72°C for 5 minutes. The reaction was cooled to 4°C, then primers ext F2 and ext R2 were added to give final concentrations of 1 µM each primer, in a final reaction volume of 50 µl. The reactions were then subjected to a denaturation step at 95°C for 1 minute, followed by 35 cycles of PCR as described above. The PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen, UK) following the manufacturer's instructions, eluted in 50 µl nuclease-free water and visualised by agarose gel electrophoresis to confirm the final size of the amplification product. The purified PCR product was cloned using the pGEM-T Easy Vector system according to the manufacturer's instructions, and the cloned inserts were amplified by PCR using primers M13For and M13Rev. The PCR products were cleaned up using the QIAquick PCR Purification Kit before being sent for sequencing at Eurofins MWG Operon.

Table 7.1. Primers used in overlap extension PCR for construction of the LAMP competitor.

Primer	Sequence (5′-3′)
ext F2	CTCTGACCAAATCATGGGC
ext R2	TCTTCGTTTCTATCTTCCTCACCT
mod F1[a]	TGAGGGGAGGTCACCTTGCAATGAGTGGCCAGC
mod R1[a]	AAGGTGACCTCCCCTCACCGGGGACAGATTCTGC

[a]Overlap region is underlined, bases for sequence modification are shown in red.

LAMP with AB-Q probe

LAMP was carried out in 25 µl reactions using *Bst* polymerase and primers for detection of *B. cinerea* as described by Tomlinson et al. (2010b). Reactions contained 0.32 U μl^{-1} *Bst* DNA polymerase (New England Biolabs, Ipswich, MA), 1 x Thermopol buffer (supplied with *Bst* polymerase), 1.4 mM each dNTP, 6 mM $MgSO_4$ (including 2 mM in the Thermopol buffer), 1.2 M betaine, 200 nM each external primer (F3 and B3), 2 µM each internal primer (FIP and BIP), 1 µM F-loop primer (B-Loop was omitted) and 200 nM AB-Q probe. In addition, 0.5 µl ROX passive reference dye (Life Technologies, CA, USA) was added per 25 µl reaction. LAMP reactions contained 1 µl target DNA and/or 1 µl competitor DNA as required. Target DNA consisted of DNA extracted from *B. cinerea* in culture or *B. cinerea*-infected plants. The competitor consisted of the cleaned up PCR product as described above, and was used at various dilutions from a starting concentration of approximately 10 ng μl^{-1}. Incubation and fluorescence measurement was carried out using a 7900HT instrument (Life Technologies). Reactions were incubated at 58°C for two minutes with fluorescence monitoring, 65°C for 70 minutes, 95°C for 30 s, and finally 58°C for 3 minutes with fluorescence monitoring. For each reaction, fluorescence levels at 58°C before and after amplification were measured as mean value of up to 14 consecutive fluorescence measurements (fluorescence results were presented as a series of measurements collected in real-time by the 7900HT software). Tani et al. (2007b) state that the ratio of target to competitor DNA, R, can be calculated as $R = (Q - Q_C) / (Q_T - Q_C)$, where Q is the observed quench rate (fluorescence after amplification / fluorescence before amplification), Q_C is the quench rate for reactions

containing only competitor and Q_T is the quench rate for reactions containing only target. For the purposes of initial testing, however, LAMP / AB-Q probe results were interpreted in terms of quench rate only. In most cases, reactions were carried out individually within runs but replicated in more than one run.

Real-time PCR

TaqMan real-time PCR for *B. cinerea* was carried out in 25 µl reactions on an ABI 7900HT instrument using the assay developed by Suarez et al. (2005). Reactions contained 1x Buffer A (Life Technologies), 5.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.625 units AmpliTaq Gold (Life Technologies), 300 nM forward primer Bc3F (GCTGTAATTTCAATGTGCAGAATCC), 300 nM reverse primer Bc3R (GGAGCAACAATTAATCGCATTTC) and 100 nM TaqMan probe Bc3P (FAM-TCACCTTGCAATGAGTGG-MGB). Cycling conditions were 95°C for 10 minutes followed by 40 two-step cycles of 95°C for 15 s and 60°C for 60 s. Real-time PCR results were interpreted in terms of Ct values.

RESULTS

Competitor and AB-Q probe design

The size of the competitor generated by the overlap extension method was confirmed to be approximately 635 bp by agarose gel electrophoresis; furthermore, the sequence at the position of the modification, and at the LAMP primer binding sites, was confirmed by cloning and sequencing the PCR product (data not shown). Figure 7.2 shows typical results indicating the different quench rates observed for LAMP / AB-Q probe reactions containing target (*B. cinerea* DNA) or competitor. Quench rates of approximately 0.37 and 0.76 were observed for reactions containing only target and reactions containing only competitor, respectively. These values are similar to those reported by Tani et al. (2007b), and indicate that some quenching occurs on binding of the AB-Q probe to the competitor but this is less than the degree of quenching when the probe binds to the target sequence.

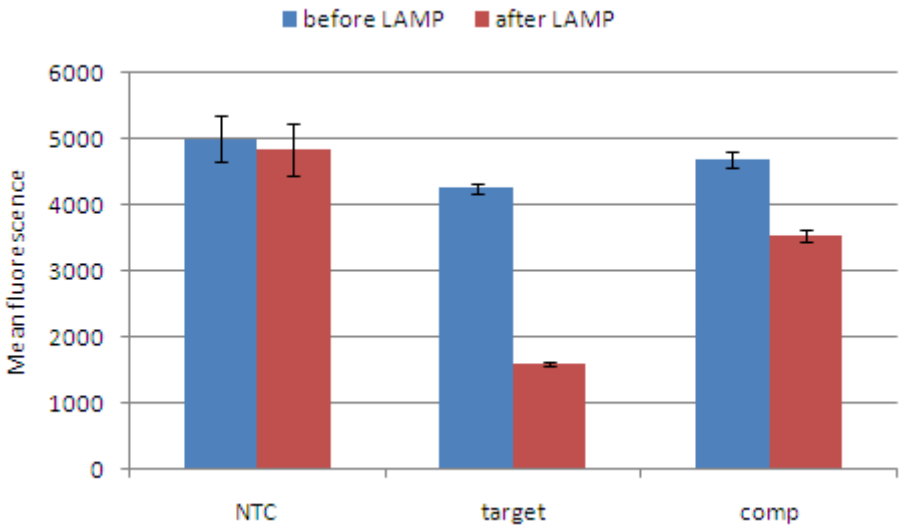

Figure 7.2. Fluorescence measurement before and after amplification of target (*Botrytis cinerea* DNA) and competitor showing differential quenching of AB-Q probe fluorescence. NTC = no template control. Fluorescence values were taken as the mean of 14 consecutive measurements on the 7900HT; figure shows mean values for duplicate reactions, error bars show standard deviation.

Correlation between quench rate and competitor concentration

In order to investigate the effects of competitor concentration, fluorescence values before and after amplification were measured and quench rates were calculated for LAMP reactions containing *B. cinerea* DNA plus different dilutions of competitor. As shown in Figure 7.3, the observed quench rate was proportional to the amount of competitor (log scale), with an R^2 value of approximately 0.96, for concentrations spanning three orders of magnitude.

Correlation between quench rate and target concentration

Quench rates were calculated for LAMP reactions containing dilutions of *B. cinerea* DNA in the presence of competitor at different concentrations. Typical results for two competitor concentrations are shown in Figure 7.4. For both competitor concentrations shown (approximately 1 pg μl^{-1} and 40 fg μl^{-1}), quench rate was proportional to the target DNA concentration (R^2 values >0.99) within a particular dynamic range, beyond which the relationship was not linear. In fact, over a broad range of target concentrations, the relationship between target concentration and quench rate is more accurately described by a rectangular hyperbola (Tani et al. 2007c). The range over which the response was approximately linear was observed to be different for each level of competitor.

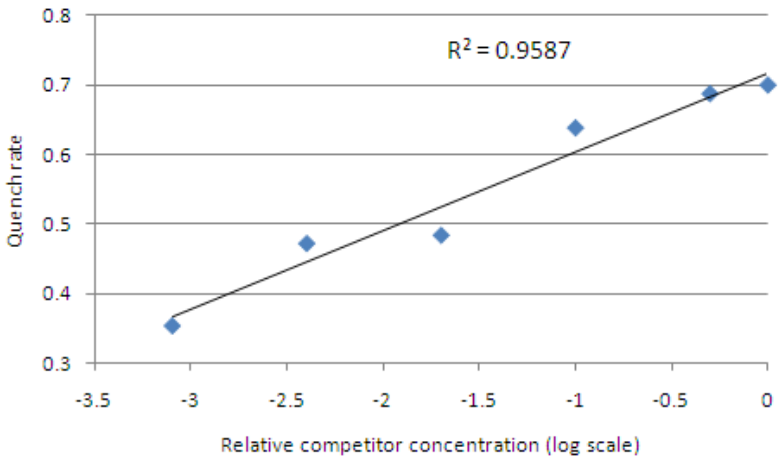

Figure 7.3. Quench rates (fluorescence after amplification / fluorescence before amplification) for reactions containing equal amounts of target (*Botrytis cinerea* DNA) and different amounts of competitor.

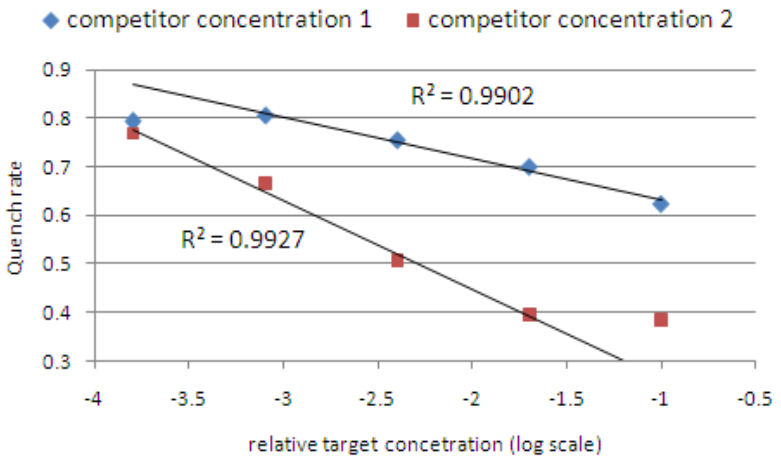

Figure 7.4. Quench rates for reactions containing two different amounts of competitor (concentration 1 = approximately 1 pg μl^{-1}; concentration 2 = approximately 40 fg μl^{-1}) plus dilutions of target (*Botrytis cinerea* DNA).

Real-time PCR and LAMP with AB-Q probe for DNA extracted from *B. cinerea* infected plants

DNA extracts from four pelargonium and rose samples containing different amounts of *B. cinerea*, dilutions of these extracts, and dilutions of DNA extracted from *B. cinerea* in culture (14 samples in total) were selected to cover a range of *B. cinerea* DNA concentrations (with and without host plant DNA), and were tested for *B. cinerea* by both LAMP with AB-Q probe and TaqMan real-time PCR. Of the 14 samples tested, one was positive by LAMP (quench rate approximately 0.5) but was negative by real-time PCR. Dilutions of this extract were positive by both methods, suggesting that the undiluted extract contained substances which completely inhibited real-time PCR but not LAMP. Figure 7.5 shows real-time PCR Ct values and quench rates for the remaining 13 samples. The lack of correlation between Ct values and quench rates is likely to reflect not only differences in the accuracy of the two methods, but also differences in susceptibility to inhibition caused by substances co-extracted from infected plant material, and potentially also differences in the linear ranges of the two methods.

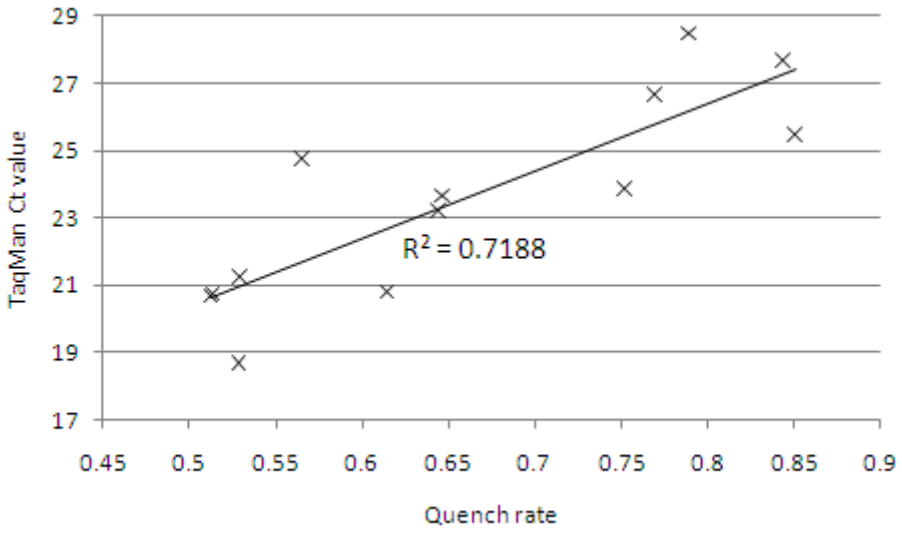

Figure 7.5. Typical results showing TaqMan real-time PCR Ct values and LAMP / AB-Q probe quench rates for extracts from a range of samples including infected plants containing different levels of *B. cinerea* DNA and dilutions of DNA extracted from culture.

DISCUSSION

Further testing is required to more fully evaluate the potential utility of the LAMP / AB-Q probe method for quantification of plant pathogens, as the data presented here represent the results of preliminary testing only. On the basis of these results, however, some general observations can be made regarding the potential for LAMP with AB-Q probes to be used for on-site quantification of plant pathogens. This method requires the design and construction of a competitor consisting of approximately 600 bp of double stranded DNA differing from the target sequence by only a small number of bases. The protocol described here for generation of a competitor by overlap extension PCR is conceptually simple but fairly laborious. Having constructed the competitor, optimisation of its concentration in the LAMP /AB-Q probe assay is also relatively time consuming. The ability to rapidly design and deploy new assays is an advantage of nucleic acid-based testing in comparison with, for example, serological tests, but it is clear that the development and optimisation of LAMP / AB-Q probe assays for new targets would be more time consuming than the development of conventional LAMP assays. Another potential disadvantage is the limited dynamic range observed for the LAMP / AB-Q probe method for a given competitor concentration (see Figure 7.4). In order to achieve quantification over a wider dynamic range it might therefore be necessary to test samples in parallel in reactions containing different levels of competitor, which would increase the complexity of the method, and in particular the interpretation of results. This could negate a potential advantage of this method over methods based on real-time detection, which is the ability to quantify targets without reference to a standard curve. Having said this,

methods could be developed for semi-quantitative detection (for example, categorisation of pathogen levels as high / medium / low, or above / below a threshold) using a single competitor concentration, as long as careful optimisation was carried out to establish the required dynamic range and to select an appropriate competitor concentration. Quantitative detection of plant pathogens for many practical applications requires only semi-quantitative results, and for some of these applications (for example, in disease management or the detection of storage pathogens) methods based on real-time monitoring may be deemed unsuitable on the grounds of instrumentation cost and complexity. AB-Q LAMP could therefore represent a viable alternative to other quantitative detection methods for some of these applications.

A preliminary comparison of quantitative results obtained by AB-Q LAMP and real-time PCR highlighted potential pitfalls in comparing the performance of quantitative methods which differ in terms of characteristics such as susceptibility to inhibition, limit of detection and dynamic range. Problems can arise when comparing the performance of a new method to a standard method which is known to be flawed. This is compounded in this case by the fact that each of the two methods can outperform the other for certain samples. For samples containing inhibitors, real-time PCR is likely to underestimate the level of pathogen, or may even fail. Conversely, for samples containing very low levels of pathogen, the LAMP / AB-Q probe method may underestimate the pathogen level or may fail because the *B. cinerea* LAMP assay is less sensitive than real-time PCR. A more informative comparison therefore requires testing of samples for which the pathogen level has been established unambiguously using other methods. Further testing of a greater

number of samples will also be required to allow a statistical examination of the relative accuracy of quantification using different PCR- and LAMP-based methods.

ACKNOWLEDGMENTS

This work was funded by Defra Seedcorn.

CHAPTER 8 GENERAL DISCUSSION

The term on-site detection can be used to refer to testing in a diverse range of circumstances, with the common feature being a lack of conventional laboratory equipment. This encompasses scenarios ranging from genuine field conditions, where basic features such as shelter and a power supply may be unavailable, to rudimentary laboratories lacking the more elaborate equipment associated with modern molecular testing facilities. The potential end-users of on-site testing methods range from diagnosticians with specific molecular biology training carrying out testing in under-resourced laboratories, to Plant Health and Seeds Inspectors or quality control personnel, who may need to deploy nucleic acid-based detection methods as just one of the many tools required in their daily activities. The requirements of these end-users could differ significantly. In this context, on-site testing methods that have been developed with a specific application and end-user in mind stand a better chance of meaningful deployment in the long term than methods which have been devised in light of more generic requirements.

Before a specific workflow for a test can be devised, the basic characteristics of the assay on which the test is based must be established. Accurate performance of the assay is a prerequisite for any testing method, with the aspiration to reproduce the detection capability of established laboratory methods. In the development of new laboratory tests it is a reasonable expectation that the new method should perform at least as well as the current method. Validation is therefore often performed with reference to a ‘gold standard’ method (although problems can arise if the standard method

has known shortcomings or if the new method systematically outperforms the existing method). The tendency is to describe the performance of new methods for on-site nucleic acid-based testing in terms of the performance of existing nucleic acid-based tests, which in most cases will be conventional or real-time PCR. Care should be taken not to emphasise this comparison too much, as a more pertinent comparison is with existing methods which can be used in the same non-laboratory conditions. In a limited number of cases this could be on-site real-time PCR, but in most cases the only on-site testing methods available are LFDs, the performance characteristics of which are likely to be very different to nucleic acid-based methods. The development of on-site methods will also allow testing to be carried out in situations where testing is not currently possible, and the value of the test will be most usefully assessed by considering the costs and benefits of testing (potentially with an imperfect method) in comparison with the current situation in which no testing at all is carried out.

Having established the performance characteristics of an assay and the potential benefits of its deployment, the specific requirements of the application and the end-user should be taken into account to shape the final configuration of the test and the associated workflow. Some aspects, particularly of the extraction method, will be primarily determined by the nature of the samples. For example, if samples are to be selected on the basis of observed symptoms, it will be possible to test small amounts of material, such as individual citrus black spot lesions (Chapter 5), without compromising sensitivity, since the pathogen is concentrated within the lesion. The small sample size and high pathogen level within the lesion are conducive to the use

of crude extraction methods. Conversely, to detect low levels of pathogen in asymptomatic material (Chapter 4), it may be necessary to test larger or bulked samples (Chapter 3) and additional steps may be required in the extraction process.

Methods for use by inspectors at import or in other scenarios will require particular emphasis to be placed on the complexity of workflows and the time taken to obtain results. Methods which are too time consuming or which require numerous manipulations are unlikely to be used in the course of routine inspection activities, with end-users likely to choose to submit samples to the laboratory for testing in spite of the delay in obtaining results. An additional issue in the context of testing for notifiable plant diseases arises in the case of pathogens for which the detection methods to be used are prescribed by EU Council Directives, as is currently the case for ring rot and brown rot of potato. Where there is a requirement for the final diagnosis to be obtained using a specified laboratory-based method, on-site detection may still have a role in the early stages of identifying cases of the disease, and would have an additional benefit of potentially increasing the credibility of the initial observation, in advance of the more extensive testing required by legislation.

In contrast to the detection of quarantine targets, methods for detection of non-quarantine pathogens may require tests that generate quantitative results. Methods for quantitative detection based on real-time detection (Chapter 4) or competitive amplification (Chapter 7) both show potential for on-site use, but additional work is required to incorporate these approaches into workflows which are as simple and user-friendly as qualitative methods. For maximum benefit, it will be necessary to establish the relationship between

pathogen levels and disease for each pathogen, and furthermore to devise means to integrate the results of quantitative testing with information about other relevant factors, such as weather conditions.

For testing in low-resource settings, the issue of cost is likely to be a more important driver than the speed of result or the simplicity of the workflow *per se*. In particular, instrumentation costs above a certain threshold will restrict uptake to well-resourced laboratories. Small instruments for isothermal amplification such as the Genie II are potentially more accessible, and bring additional benefits, not least the reduced contamination risk associated with homogeneous real-time detection. However, non-instrumented methods such as LFD detection (Chapters 3 and 6) should not be discounted, as they increase the accessibility of nucleic acid-based testing to a greater number of users. The specific issue to be addressed for non-instrumented detection will be the ability to control contamination by devising methods for closed-tube detection. An emphasis on development of simple workflows contributes to this indirectly, since methods which entail few manipulations, and which use mostly disposable components, can be more easily segregated to prevent carry-over contamination with amplification products and cross-contamination between samples.

Applications for detection methods in the context of inspection or industry (horticulture, agriculture and processing of plant products) will have specific per-test cost requirements, informed by cost-benefit analysis for each application, and methods for deployment in low-resource settings will also have a maximum cost above which the use of a test becomes unfeasible. The per-test cost is a critical factor in influencing how and when a test is used,

regardless of the accuracy of the method or the pertinence of the information it provides. Issues of licensing and availability of reagents will be crucial in determining the uptake of LAMP-based on-site testing. LFDs for plant pathogens typically cost in the region of £5 to 10, and the cost of the individual components of a LAMP reaction, plus reaction tubes and basic consumables for a crude extraction, would typically amount to less than this, such that the per-test cost of a commercial LAMP-based kit might be expected to be comparable to the per-device cost for LFDs. For *ad hoc* testing in the field LFDs are commonly run without replication and without controls. LAMP-based methods, however, are more sensitive and therefore liable to contamination, so the use of negative controls is necessary for proper interpretation of results. Furthermore, since nucleic acid-based methods consist of more individual components (if not more manipulations), which could each fail due to user error or improper storage, it is also necessary to run positive controls and, in some cases, replicate reactions. Replication of reactions and the use of controls increase the per-sample cost, so that while the per-reaction cost of LAMP may be less than the price of an LFD, to obtain a result by LAMP may be more expensive than testing the same sample by LFD.

Regardless of the specific application, the aims of deploying detection methods outside the laboratory are to increase efficiency of testing and to extend the reach of pathogen detection capabilities. Increasing efficiency will ultimately have the effect of maximising the impact of testing. On-site detection of pathogens enables decisions to be taken more rapidly in the field and also has the potential to allow resources and equipment to be used more efficiently in the laboratory by reducing the number of samples sent there to be

processed. Performing nucleic acid-based testing at inspection also has the potential to add value to the inspection process and reinforce the credibility of decisions regarding action to be taken.

Another significant way in which the use of nucleic acid-based testing can increase the efficiency with which plant diseases are controlled is in increasing responsiveness of NPPOs to new and emerging threats. Capability to deploy on-site testing methods without having to rely solely on laboratory testing (although integration with laboratory processes is a key issue) will expedite action in response to outbreaks, allowing control measures to be implemented to best effect. Perhaps even more significantly, the use of nucleic acid-based methods allows greater responsiveness to new and emerging threats, since primers for the detection of new targets can be developed in a period of days, in contrast to new monoclonal antibodies or polyclonal antiserum, which can take many months to produce. The amount of sequence data available in publicly accessible databases is growing at a rapid pace; furthermore, next generation sequencing technology now provides the capability to generate complete or almost complete genomes extremely rapidly, so that, even if no sequence data for an organism exists, the processes to generate large amounts of sequence on which primer design can be based are rapid and becoming routine. Beyond generating sequence data which can be used for assay design, next generation sequencing also has a role to play in the discovery of new pathogens and the ability to link them to diseases of previously unknown aetiology (Adams et al. 2009). In the context of managing the threat posed by newly characterised pathogens, the development of methods for their routine detection is the logical next step and is expedited by

the availability of large amounts of reliable sequence information. A novel approach to assay design is the use of comparative genomics to identify diagnostic regions for LAMP primer design (Li et al. 2009: Li et al. 2011; Bühlman et al. 2012). With the advent of next generation sequencing, and the availability of genome sequences for increasing numbers of organisms, it is likely that this approach will become more common in the rapid development of assays with optimal performance characteristics. Software for LAMP primer design is available, but to generate highly specific assays it is often necessary to design primers manually in order to exploit the small sequence differences that can exist between species in the conserved gene regions that are most represented in sequence databases. Comparative genomic approaches have the potential to identify putative diagnostic regions with no significant homology to any known non-target sequences; these regions can then be used as the input sequences for primer design software, which usually results in the design of very reliable assays. Ultimately, the establishment of pipelines for assay design, such that putative diagnostic regions can be identified for target species through genomic comparisons with related non-target species in a semi-automated fashion, will expedite the development and deployment of assays. In the short term, ongoing efforts to systematically collect and curate sequence information for as many species as possible (an approach referred to as DNA barcoding) are generating sequence information which will be a useful resource for assay development, even if the barcoding regions are not ultimately the best candidates for primer design. Having established pipelines for the rapid development of new assays, and with a portfolio of nucleic acid extraction methods and amplicon detection technologies that can be combined

as required, it will be necessary to have in place mechanisms to rapidly establish and demonstrate the validity of new detection methods, such that the results of testing, and the actions taken on the basis of those results, have maximum credibility. Adherence to international guidelines for validation of new methods will ensure that maximum value can be gained from the deployment of on-site testing for plant pathogens.

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