

Development of a LAMP assay for *Xylella fastidiosa* (2009 - Int002)

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EXECUTIVE SUMMARY

Xylella fastidiosa is a regulated plant pathogen in many parts of the world. To increase diagnostic capability of *X. fastidiosa* in the field a loop-mediated isothermal amplification (LAMP) was developed to the *rimM* gene of *Xylella fastidiosa*, and evaluated for specificity, sensitivity and reliability. The assay was more robust than existing published assays for detection of *X. fastidiosa* when screened against 20 isolates representing the four major sub-groups of the bacterium, and a range of host species. No cross-reaction was observed with DNA from healthy host tissue or other bacterial species. The LAMP assay could detect as little as 250 copies of the *rimM* gene. The LAMP assay was also found to have greater sensitivity than conventional PCR, if less than a published real-time PCR assay.

A series of endpoint detection methods, including fluorescent and colorimetric dyes, and lateral flow devices were also assessed. It was found that a colorimetric dye, hydroxynaphthol blue performed best and could indicate the presence of 500 copies of the template with a purple to blue colour change after 60 minutes incubation; hydroxynaphthol blue was also found to be the most suitable for field use as the reaction could be completed in a closed-tube format.

Techniques suitable for DNA extraction from plant tissue and insect *in situ* were compared to a standard silica-column-based laboratory extraction method and it was found that a portable PickPen[®] and magnetic bead system can be used to successfully extract DNA from infected tissue and could be used in conjunction with LAMP in the field, whilst *X. fastidiosa* was successfully amplified from insect tissue extracted using the commercial PrepGEM extraction kit.

1. Literature Review

Deliverable 1: Review literature to identify LAMP assays which have been developed previously for other pathogens (including associated nucleic acid extraction methods), and previously published molecular assays for Xylella fastidiosa.

1.1. LOOP MEDIATED ISOTHERMAL AMPLIFICATION

Loop mediated isothermal amplification (LAMP) (Notomi *et al.* 2000; Nagamine *et al.* 2002) is a relatively recent and novel technique for the specific amplification of DNA templates at a single temperature, hence requiring less specialised equipment than conventional PCR technologies. This technique has been used to detect several vertebrate and plant pathogens, including bacteria and viruses (Fukuta *et al.* 2004; Savan *et al.* 2004; Dukes *et al.* 2006; Varga and James 2006; Tomlinson *et al.* 2007), and shows promise as a rapid and effective method for the detection of important pathogens (Tomlinson and Boonham 2008).

1.1.1 The LAMP reaction

The basis of the LAMP method is the strand displacement activity of the *Bacillus subtilis* derived *Bst* DNA polymerase with specific activity between 60-65°C. Many other polymerases, including the bacteriophage Φ 29 and *Escherichia coli* DNA polymerase I, display strand displacement activity although at significantly lower temperature optima which exclude their use in a LAMP reaction. LAMP operates through the priming of heat-denatured positive and negative strands with two template-specific inner primers on which the *Bst* polymerase binds and releases single-strand DNA. This is then targeted by outer primers from which template extension, or more simply, second strand synthesis can occur. LAMP differs from normal PCR in that the 3' and 5' ends of the inner primers bind to two different, though neighbouring regions within the template, with a small, generally 4-mer non-specific oligo 'hinge' between, that when bound to ssDNA template forms a 'dumbbell-like' shape that initiates further extension. An additional two loop primers trigger secondary strand displacement reactions within extended template (Nagamine *et al.* 2002; Fukuta *et al.* 2004). As the reaction proceeds the DNA template resembles a cauliflower-like shape with multiple copies joined by the loop-forming inner primers, and being displaced by outer and loop primer pairs. The end result of strand-displacement, loop formation and synthesis is the single-temperature amplification of a highly-specific fragment from a DNA template at far greater titre than an equivalent PCR reaction (Notomi *et al.* 2000; Nagamine *et al.* 2002; Tomlinson and Boonham 2008).

1.1.2 Comparison to conventional and real-time PCR

The LAMP reaction differs in several major aspects to conventional PCR amplification and its variants. The use of six primers provides a greater level of target specificity than can be achieved with the two primers normally used in PCR (Notomi *et al.* 2000); this is both an advantage and a shortcoming of the LAMP process in that polymorphism, particularly at the 3' end of the F1 and 5' end of the B1 loop primers within the target organism can reduce the likelihood of a successful amplification (Dukes *et al.* 2006). LAMP is however, less susceptible to interference from non-target DNA presence in the reaction, comparable to nested PCR (Notomi *et al.* 2000).

The sensitivity of LAMP for detection of low copy number templates, ie: less than 100 copies/reaction, is largely dependent on the target selected, template purity and the presence

of inhibitory substances (Notomi *et al.* 2000). The reported template titres for which LAMP has been achieved has been as low as 6 copies/reaction for pure template, although a minimum of 60 copies is required in the presence of a total genomic extract (Notomi *et al.* 2000) and approximately 300 copies/reaction in *Citrus* samples (Okuda *et al.* 2005). Comparative assays with standard and real-time PCR protocols have produced widely differing results with little standardisation, for example 10 copies/reaction of a plasmid template was amplified by both LAMP and real-time PCR, although in practice the same LAMP assay failed to amplify 12 of 44 samples positive by real-time PCR (Dukes *et al.* 2006). There is a general consensus that LAMP is less sensitive than real-time PCR and nested PCR, but more sensitive than standard PCR (Fukuta *et al.* 2004; Savan *et al.* 2004; Okuda *et al.* 2005; Dukes *et al.* 2006; En *et al.* 2008; Tomlinson and Boonham 2008), although one report suggests that LAMP is less sensitive than standard PCR in the presence of inhibitory substances, with LAMP failing to detect less than 10^8 CFU/ml of *Salmonella enterica* in milk, while standard PCR detected 10^6 CFU/ml (Wang *et al.* 2008).

Finally, unlike normal PCR, LAMP does not require a dedicated thermocycler and reactions can readily be achieved in standard heat-block or water-bath apparatus (Notomi *et al.* 2000; Savan *et al.* 2004; Tomlinson and Boonham 2008). In addition, a number of methods, discussed in section 1.1.3, for detection of successful LAMP reactions do not require electrophoresis or fluorescence-detection equipment (Tomlinson and Boonham 2008).

1.1.3 Detection of LAMP products

The result of LAMP reactions may be detected using gel electrophoresis of the samples as per standard PCR, which reveals the characteristic ladder pattern of LAMP amplification (Tomlinson and Boonham 2008). The high titre of product produced during the LAMP process offers alternatives for amplification product detection including intercalating dyes (Iwamoto *et al.* 2003; Dukes *et al.* 2006; Tomlinson and Boonham 2008) and by-products from the reaction chemistry (Mori *et al.* 2001; Goto *et al.* 2009).

Intercalating dyes include SYBR green and Picogreen, both of which intercalate into any double-stranded DNA present (Iwamoto *et al.* 2003; Dukes *et al.* 2006; Tomlinson *et al.* 2007). Both dyes can be scored visually (Dukes *et al.* 2006; Tomlinson *et al.* 2007), or by measurement in a real-time PCR machine or equivalent fluorometer (Dukes *et al.* 2006). However, it should be noted that the intercalating dyes are incapable of discrimination of target and non-target synthesis, therefore it is important that new assays are thoroughly validated before use to ensure the assay is specific to the target being detected. Also, it should be noted that LAMP procedure is an endpoint protocol, similar to standard PCR, and does not lend itself to accurate quantification in the manner of a real-time PCR.

Two other alternatives have been proposed for the detection of positive LAMP reactions, based on chemical changes or by-products of the amplification process. Detection of Magnesium pyrophosphate, a by-product of DNA synthesis is achieved by the addition of Tth-pyrophosphatase which increases the turbidity of the reaction by precipitation, allowing detection visually or more commonly, by spectrophotometer (Mori *et al.* 2001). Another method recently proposed is the addition of hydroxyl-naphthol blue, a chelating agent that changes colour as the concentration of Mg^{2+} ions, which are required for polymerase activity, decreases (Goto *et al.* 2009).

1.1.4 Nucleic acid template extraction techniques for LAMP

It has already been noted that the LAMP procedure is susceptible to inhibition in some substrates (Wang *et al.* 2008), such as milk in which the calcium ions compete with magnesium, both divalent cations, for binding sites on the DNA polymerase (Bickley *et al.* 1996). This is less of a problem with samples of plant origin, although the complex and abundant polysaccharides in plant material are known to inhibit polymerase activity (Demeke and Adams 1992). In addition, environmental contaminants such as an humic acid-like inhibitor from soil (Watson and Blackwell 2000) have been shown to inhibit polymerase activity.

Table 1.1: Survey of extraction methods applied to successfully extract nucleic acids for LAMP amplification from the literature.

| Method | Nucleic Acid | Template | Reference |
|---|--------------|--|--------------------------------|
| Commercial nucleic acid column-based kits | RNA | <i>Picornavirus</i> from epithelial cell culture | (Dukes <i>et al.</i> 2006) |
| | | <i>Japanese yam mosaic virus</i> from yam. | (Fukuta <i>et al.</i> 2003) |
| | | <i>Plum pox virus</i> from <i>Prunus</i> sp. | (Varga and James 2006) |
| | DNA | <i>Ralstonia</i> , <i>Enterobacter</i> , and <i>Erwinia</i> from cell culture. | (Kubota <i>et al.</i> 2008) |
| | | <i>Pseudorabies virus</i> from brain tissue | (En <i>et al.</i> 2008) |
| CTAB | DNA | <i>Phytophthora ramorum</i> cell culture | (Tomlinson <i>et al.</i> 2007) |
| PBS-Tween (with Immunocapture) | RNA | <i>Candidatus Liberibacter asiaticum</i> from <i>Citrus</i> sp. | (Okuda <i>et al.</i> 2005) |
| Heat lysis | DNA | <i>Tomato spotted wilt virus</i> from <i>Dendranthema grandiflora</i> . | (Fukuta <i>et al.</i> 2004) |
| | | <i>Edwardsiella tarda</i> cell culture | (Savan <i>et al.</i> 2004) |
| | | <i>Shigella</i> sp. cell culture | (Song <i>et al.</i> 2005) |

The method used to extract template for the LAMP process varies depending on the source material and whether RNA or DNA is required for the procedure. Extraction methods used successfully with different templates are shown in Table 1. It can be seen that commercial column based kits are most frequently used as they provide clean total DNA or RNA preparations with a minimum of inhibitor presence, and have been used successfully from microbial cell cultures (Tomlinson *et al.* 2007; En *et al.* 2008; Kubota *et al.* 2008), animal tissue culture (Dukes *et al.* 2006), and from plant host species (Fukuta *et al.* 2003; Varga and James 2006). However, a crude CTAB method has been used to successfully extract the citrus greening organism from *Citrus* species (Okuda *et al.* 2005), a host replete with inhibitors (Harper, *unpublished*). Similar crude heat lysis methods have been applied to bacterial species (Savan *et al.* 2004; Song *et al.* 2005). Interestingly, immuno-capture RT-LAMP has been successfully applied to virus detection in chrysanthemum samples, suggesting that very simple preparations may be sufficient. It has been stated that LAMP is less susceptible to inhibitor carry-over than standard PCR (Dukes *et al.* 2006), although it remains to be ascertained whether crude, field-ready extraction procedures will extract low titre pathogens reliably for detection, given the lower limit of LAMP sensitivity.

1.2. XYLELLA FASTIDIOSA

Xylella fastidiosa is a gram-negative gamma-proteobacterium that causes severe chlorosis, wilt and dieback symptoms on species as diverse as grapevine, citrus, almond, coffee and oleander (Hopkins and Purcell 2002). There are reported to be over 145 recognised strains of *X. fastidiosa*, although these have been generally subdivided into groups on the basis of host

range, random amplified polymorphic DNA (RAPD) analysis, sequence of the 16S-23S ribosomal RNA gene sequences, and more recently, complete genome analysis (Pooler and Hartung 1995; Chen *et al.* 2000; Henderson *et al.* 2001; Rodrigues *et al.* 2003b; Van Sluys *et al.* 2003; Doddapaneni *et al.* 2006). Host range has been used for differentiation, although it has been found that several grape-infecting strains also cause Almond leaf scorch and Alfalfa dwarf diseases, while another group includes strains that cause Phony peach, Plum leaf scald, Citrus variegated chlorosis and Coffee leaf scorch (Hopkins and Purcell 2002). There are also a number of strains or subtypes that infect elm, maple, oak, oleander, periwinkle, pear and sycamore species (Henderson *et al.* 2001; Hopkins and Purcell 2002; Blexine and Child 2007; Chen *et al.* 2008; Huang 2009). Cross-infectivity of strains is not certain, with peach strains not causing disease in grape and vice-versa, while both grape and oleander strains infect almond (Hopkins and Purcell 2002).

1.2.1 Current methods for detection of *Xylella fastidiosa*

Xylella fastidiosa may be detected by traditional symptom and culture-based techniques (Rodrigues *et al.* 2003b), however, it is difficult to culture and in *Vitis* sp. can remain asymptomatic throughout much of the year (R. Alameida, *personal communication*). Molecular techniques include PCR (Huang 2009) and PCR derivatives including RFLP and RAPD analysis (Pooler and Hartung 1995), as well as real-time PCR (Schaad *et al.* 2002; Francis *et al.* 2006; Blexine and Child 2007) and more recently, sequence analysis (Chen *et al.* 2000; Henderson *et al.* 2001; Rodrigues *et al.* 2003b; Van Sluys *et al.* 2003; Doddapaneni *et al.* 2006; Chen *et al.* 2008).

Extraction of *X. fastidiosa* DNA from culture and host species for PCR and related molecular analyses has been achieved from tissue by both standard commercial column kits (Blexine and Child 2007; Huang 2009) and by basic CTAB (Henderson *et al.* 2001; de Souza *et al.* 2003; Rodrigues *et al.* 2003b) or, in the case of cultures, Tris-EDTA-Sarkoysl (Pooler and Hartung 1995; Chen *et al.* 2000; Francis *et al.* 2006) techniques.

2. Collection of *Xylella fastidiosa* samples

Deliverable 2: Collect plant material and insects (at least two species of each) infected with strains of Xylella fastidiosa, including those infecting grapevine and at least one other host.

Xylella fastidiosa cultures were obtained from commercial (DSMZ, Mannheim, Germany) and academic sources (Landcare Research, Auckland, New Zealand). Lyophilised *X. fastidiosa*-infected samples of *Vitis vinifera*, *V. rotundifolia* and *Quercus rubra* leaves, and infected blue-green sharpshooters (*Graphocephala atropunctata*) were obtained from Dr. R. Almeida (University of California, Berkley, CA, USA) and C. Chang (University of Georgia, USA). DNA samples of *X. fastidiosa* extracted from a range of host species were obtained either on FTA cards (Whatman Inc., Florham Park, NJ, USA) or lyophilised, from Dr. L. Nunney (University of California, Riverside, CA, USA), Dr. C. Su, (Agricultural Chemicals Toxic Substances Research Institute, Taichung, Taiwan) and Dr. H. Coletta Filho, (Centro de Citricultura, Cordieropolis, Brazil). *Spiroplasma citri* DNA was obtained from Dr. R. Yokomi (United States Department of Agriculture, Parlier, CA, USA). Finally, DNA of healthy host-plant species and non-target bacterial species *Xanthomonas campestris* pv. *citri*, *Xanthomonas axonopodis* pv. *aurantifolii* was obtained from the MAF Biosecurity New Zealand nucleic acid collection. Samples collected are shown in Table 2.1, sorted by subspecies where known.

Table 2.1: Samples collected to assess the sensitivity and specificity of the *Xylella fastidiosa* LAMP assay.

| Species | Host | Isolate | Country of Origin | Source |
|---|--|---------|------------------------|-------------------------------|
| <i>X. fastidiosa</i> subsp. <i>fastidiosa</i> | <i>Vitis vinifera</i> | | Unknown | DSMZ cat. 10026 |
| | <i>Vitis vinifera</i> | | Unknown | Sediag cat. Xylf-LPC |
| | <i>Vitis vinifera</i> | | Unknown | Loewe cat. 07119pc |
| | <i>Vitis vinifera</i> | PD0001 | USA | L. Nunney, UC Riverside |
| | <i>Vitis vinifera</i> | PD0004 | USA | L. Nunney, UC Riverside |
| | <i>Vitis vinifera</i> 'Cabernet Sauvignon' | | USA | R. Almeida, UC Berkley |
| | <i>Vitis vinifera</i> 'Merlot' | | USA | R. Almeida, UC Berkley |
| | <i>Prunus dulcis</i> | ALS0005 | USA | L. Nunney, UC Riverside |
| | <i>Prunus dulcis</i> | ALS0095 | USA | L. Nunney, UC Riverside |
| | <i>Prunus dulcis</i> | ALS0096 | USA | L. Nunney, UC Riverside |
| | <i>Vitis rotundifolia</i> | | USA | C. Chang, U Georgia |
| <i>Graphocephala atropunctata</i> | | USA | R. Almeida, UC Berkley | |
| <i>X. fastidiosa</i> subsp. <i>multiplex</i> | <i>Prunus salicina</i> | | USA | ICMP-8375 |
| | <i>Prunus dulcis</i> | | USA | ICMP-6575 |
| | <i>Prunus dulcis</i> | ALS0003 | USA | L. Nunney, UC Riverside |
| | <i>Quercus laevis</i> | OAK0023 | USA | L. Nunney, UC Riverside |
| | <i>Quercus rubra</i> | OAK0024 | USA | L. Nunney, UC Riverside |
| | <i>Quercus rubra</i> | | USA | C. Chang, U Georgia |
| | <i>Liquidambar styraciflua</i> | LIQ0063 | USA | L. Nunney, UC Riverside |
| <i>X. fastidiosa</i> subsp. <i>sandyi</i> | <i>Nerium oleander</i> | OLS002 | USA | L. Nunney, UC Riverside |
| | <i>Nerium oleander</i> | OLS008 | USA | L. Nunney, UC Riverside |
| | <i>Nerium oleander</i> | OLS009 | USA | L. Nunney, UC Riverside |
| <i>X. fastidiosa</i> subsp. <i>pauca</i> | <i>Citrus</i> sp. | 9a5C | Brazil | H. Coletta Filho, CDC Brasil. |
| | <i>Citrus</i> sp. | 9a5C | Brazil | H. Coletta Filho, CDC Brasil. |
| Unclassified subspecies | <i>Pyrus</i> sp. | | Taiwan | C. Su |

3. Designing LAMP primers for *Xylella fastidiosa*

Deliverable 4: Evaluate sequence data for primer design, taking into account sites used for previous molecular assays. Design a loop-mediated isothermal amplification (LAMP) assay for the detection of Xylella fastidiosa in plants and insect vectors, in collaboration with the Food & Environment Research Agency, UK.

3.1. SEQUENCE DATA

At time of writing there are approximately 2760 *Xylella fastidiosa* sequences of variable length available on the NCBI GenBank database, covering partial and complete 16S rRNA sequences to avirulence genes and markers such as *gyrB* (Rodrigues *et al.* 2003a). There are also a total of four annotated complete genomic sequences of *X. fastidiosa*, of between 2.4-2.5 MB in length, representing Citrus variegated chlorosis, Almond, Oleander and Pierce's Disease strains (Van Sluys *et al.* 2003; Doddapaneni *et al.* 2006).

Phylogenetic analysis of 32 available 16S sequences of *X. fastidiosa* from all hosts indicated 99.2 percent identity between all strains, suggesting that this is a suitable conserved region for primer design. However, it was also found that the *X. fastidiosa* 16S rRNA has an average of 96.2 percent nucleotide identity to the closely related *Xanthomonas* and *Pantoea* species; use of the proprietary LAMP design software (Eiken Chemical Co., Tokyo) indicated that no LAMP primers could be designed that would selectively amplify only *X. fastidiosa*.

There are a number of genes or regions within the *X. fastidiosa* genome that have been examined for strain typing or phylogenetic analysis (Pooler and Hartung 1995; Chen *et al.* 2000; Rodrigues *et al.* 2003a; Scally *et al.* 2005; Francis *et al.* 2006; Blexine and Child 2007; Chen *et al.* 2008), although these vary greatly in sequence conservation. More recently, Doddapaneni *et al.* (2006) assessed the four complete genomic sequences available and identified genes unique to *X. fastidiosa*, and genes that were highly conserved between all *X. fastidiosa* sequences. Cross-referencing these data identified four genes, *pdiA*, *rimM*, *hicB* and *FtsH* that could be potential candidates for primer design. Additional gene targets were chosen on the basis of sequence availability, use in published PCR assays (Francis *et al.* 2006; Blexine and Child 2007), and for novel targets, sequence conservation and specificity to *X. fastidiosa*.

A total of eight ORF or gene targets were assessed for the design of *Xylella fastidiosa* specific LAMP primers (Table 3.1), and of these, three (16S rRNA, *gyrB*, hypothetical protein) were rejected due to low sequence (<98 percent) conservation, and similarity to related genera such as *Xanthomonas*.

Table 3.1: Genes of *Xylella fastidiosa* considered for potential LAMP primer design.

| Gene | Length (bp) | Location on genome (9a5C strain) | % Nucleotide Identity between strains |
|---|-------------|----------------------------------|---------------------------------------|
| <i>16S rRNA</i> | 1536 | 65886-67422 | 96.7% |
| <i>gyrB</i> | ? | 4634-7078 | ? |
| <i>pdiA</i> | 465 | 1751131-1750670 | 99.1% |
| <i>rimM</i> | 513 | 106499-107011 | 99.3% |
| <i>hicB</i> | 336 | 1603673-1604032 | 99.7% |
| <i>gltA</i> | 853 | 933968-935257 | 99.4% |
| <i>FtsH</i> | 642 | 94878-94237 | 98.7% |
| Hypothetical Protein (Francis <i>et al.</i> 2006) | 801 | ? | 97.9 |

3.2. PRIMER AND ASSAY DESIGN

Primers for LAMP amplification were designed against the five candidate genes using the standard default parameters of the online PrimerExplorer V4 software (Eiken Chemical Co., Tokyo). Outer (F3/B3) and loop (FIP/BIP) primer pairs were designed for all five gene targets, however, due to product length and primer sites selected by the software, inner primers (LF/LB) could not be designed for the *FtsH* and *hicB* genes. Therefore, complete primer sets (Table 3.2) for *in vitro* testing were designed against the *rimM*, *pdiA* and *gltA* genes.

Table 3.2: Primer sets designed for LAMP amplification of *Xylella fastidiosa*.

| Gene target | Primer name | Sequence(5'--3') |
|---|-------------|--|
| Disulphide Isomerase (<i>pdiA</i>) | XF-DIS-FIP | CGTGGGCAATTTTGACCGCAATTTTCGGAATGCCATCCTGGATT |
| | XF-DIS-BIP | GTGCTTTGCAATCAGCGCCGTTTTAACTGGTGCACCGACTGT |
| | XF-DIS-F3 | GCCATCCGAATTCACCACAA |
| | XF-DIS-B3 | ACACACAAACCAACCCTCTT |
| | XF-DIS-LF | AGTCAGGCCTATGGCGAC |
| | XF-DIS-LB | TACGCAATGATTTATCGAGTGCTCG |
| 16S rRNA processing protein (<i>rimM</i>) | XF-RIMM-LF | TGCAAGTACACACCCTTGAAG |
| | XF-RIMM-LB | TTCCGTACCACAGATCGCT |
| | XF-RIMM-F3 | CCGTTGAAAACAGATGGGA |
| | XF-RIMM-B3 | GAGACTGGCAAGCGTTTGA |
| | XF-RIMM-FIP | ACCCCGACGAGTATTACTGGGTTTTTCGCTACCGAGAACCACAC |
| | XF-RIMM-BIP | GCGCTGCGTGGCACATAGATTTTTGCAACCTTTCCTGGCATCAA |
| Citrate synthase (<i>gltA</i>) | XF-GLTA-LF | ATCGGATAGCCGCGGTACAACA |
| | XF-GLTA-LB | GACGAGTTTGCCAAGTTTGATG |
| | XF-GLTA-F3 | GCAAGCTGTAAGTCTGCCAT |
| | XF-GLTA-B3 | ACCGCCAAGAAAGTTCTTCA |
| | XF-GLTA-FIP | GGAGTGCTCAGCTAACTGCTCATTTTTGATGGTAAAAGGGTGTGT |
| | XF-GLTA-BIP | ATGAACGGTGAGTTGCCGAGAATTTTATCATCGTGTGATGCGTGAT |

3.2.1 Preliminary primer testing

To ensure that the designed assays were viable prior to specificity testing, each LAMP assay was tested against a range of samples that were known to be positive by PCR or qPCR. LAMP was conducted using 1x ThermoPol buffer (20 mM Tris-HCl 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1 percent Triton X-100, pH 8.8) (New England Biolabs, Beverly MA), additional MgSO₄ to a final concentration of 8mM, 0.8M

Betaine, 1.4mM dNTP, 0.2 µM of outer (F3/B3) primers, 0.8 µM of inner (LF/LB) primers and 1.6 µM of the loop primers (FIP/BIP), with 8 units of *Bst* DNA polymerase (New England Biolabs), and 2 µl of DNA template in a total volume of 25 µl. The reaction was incubated at the predicted optimum 63°C for 75 minutes, and successful amplification was confirmed by agarose gel electrophoresis of a 15 µl aliquot of LAMP product.

Table 3.3: Results of preliminary testing of the three *X. fastidiosa* specific LAMP primer sets against known positive samples. '+': positive amplification, '-': negative or no amplification.

| Host | Type | | <i>pdiA</i> | <i>rimM</i> | <i>gltA</i> | |
|--|------------------------|-----------------------|---------------------|-------------|-------------|---|
| <i>Vitis vinifera</i> | Culture | DSMZ cat. 10026 | + | + | - | |
| <i>Vitis vinifera</i> | | Sediag cat. Xylf-LPC | + | + | - | |
| <i>Vitis vinifera</i> | | Loewe cat. 07119pc | + | + | - | |
| <i>Prunus salicina</i> | | ICMP-8375 | + | + | - | |
| <i>Prunus dulcis</i> | | ICMP-6575 | + | + | - | |
| | | H. Coletta Filho, CDC | | | | |
| <i>Citrus</i> sp. | | Brasil. | + | + | - | |
| <i>Pyrus</i> sp. | | C. Su | - | - | - | |
| <i>Quercus rubra</i> | | Tissue | C. Chang, U Georgia | + | + | - |
| <i>Vitis rotundifolia</i> | | | C. Chang, U Georgia | - | + | - |
| <i>Vitis vinifera</i> 'Cabernet Sauvignon' | R. Almeida, UC Berkley | | - | + | - | |
| <i>Vitis vinifera</i> 'Merlot' | R. Almeida, UC Berkley | | - | + | - | |
| <i>Graphocephala atropunctata</i> | R. Almeida, UC Berkley | | + | + | - | |

It can be seen from Table 3.3 that only the *rimM* assay successfully amplified all known *X. fastidiosa* positive samples, with the exception of Pear leaf scorch which was not amplified by any of the three primer sets. The *pdiA* assay failed to amplify several of the tissue extracts which may be the result of inhibition, whilst the *gltA* assay did not amplify any samples; both of these primer sets were discarded and the *rimM* set was selected for further testing.

4. LAMP Specificity, sensitivity and reliability

Deliverable 6: Validate the LAMP assay in terms of its sensitivity, specificity and reliability, and compare these parameters against existing conventional and real-time PCR techniques

4.1. MATERIALS AND METHODS

4.1.1 Sample collection and DNA extraction

Pure *Xylella fastidiosa* cultures, *X. fastidiosa*-infected tissue and insect vector samples, and DNA were obtained from a range of sources (Table 1.1). Healthy host-species tissue and DNA of non-target bacterial species were obtained from the MAFBNZ nucleic acid collection. Infected and healthy tissue samples were ground to a fine powder in liquid nitrogen prior to extraction using the DNeasy Plant Mini kit (Qiagen Inc., Valencia CA) as described (Huang *et al.* 2006). *X. fastidiosa* DNA samples obtained on FTA cards were eluted using the Sigma Extract-N-Amp kit (Sigma-Aldrich, St. Louis MO) as per the manufacturer's protocol.

4.1.2 LAMP amplification

The LAMP assay was conducted in 1x ThermoPol (20 mM Tris-HCl 10 mM (NH₄)₂SO₄, 10 mM KCl, 2 mM MgSO₄, 0.1 percent Triton X-100, pH 8.8) buffer (New England Biolabs, Beverly MA), additional MgSO₄ to a final concentration of 8mM, 0.8M Betaine, 1.4mM dNTP, 0.2 µM of outer (F3/B3) primers, 0.8 µM of inner (LF/LB) primers and 1.6 µM of the loop primers (FIP/BIP), with 8 units of *Bst* DNA polymerase (New England Biolabs), and 2 µl of DNA template in a total volume of 25 µl. The reaction was incubated at 65°C for 75 minutes, and successful amplification confirmed by agarose gel electrophoresis of a 15 µl aliquot of LAMP product.

4.1.3 Published assays used for comparison

4.1.3.1. Minsavage *et al.* (1994) PCR assay

Standard PCR screening of *X. fastidiosa* was conducted using the protocol of Minsavage *et al.* (1994), which targets the 16S rDNA gene. Reactions were conducted in a final volume of 20 µl, using 10 µl of 2x GoTaq polymerase (Promega, Madison WI), 250 nM each of forward and reverse primers, 500 ng/µl bovine serum albumin, and 2 µl of total DNA template. Thermocycling conditions were as follows: 94°C for three minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, then a final extension of 5 minutes. Successful amplification was confirmed by agarose gel electrophoresis of a 10 µl aliquot of reaction product.

4.1.3.2. Francis *et al.* (2006) real-time PCR assay

Real-time PCR for amplification of *X. fastidiosa* was conducted using the TaqMan[®]-based probe and primers designed by Francis *et al.* (2006) in a Corbett RotorGene 3000 (Qiagen Inc., Valencia CA) using Invitrogen qPCR Supermix-UDG (Invitrogen, Carlsbad CA) with a final concentration of 3mM MgCl₂, 250 nM of sense and antisense primers, 100 nM of FAM/BHQ labelled probe, 300 ng/µl bovine serum albumin, and 2 µl of total DNA template in a final reaction volume of 20 µl. Thermocycling conditions were as follows: 50°C for 2 minutes, 95°C for two minutes, then 40 cycles of 95°C for 10 seconds and 60°C for 45

seconds. Quantification was conducted against a serial dilution (from 10^7 to 10^2 copies / μl) of the amplification product, and threshold applied automatically.

4.1.4 Comparison of specificity

A comparison of the specificity of the LAMP assay was made to published *Xylella*-specific standard PCR (Minsavage *et al.* 1994) and real-time qPCR assays (Francis *et al.* 2006) using a series of *X. fastidiosa* isolates from the four major subspecies extracted from a range of host species, or from cell culture. *Xylella*-free host species and non-target bacterial species were also examined. All samples were tested in duplicate, and the results tabulated for comparison (Table 4.1).

4.1.5 Comparison of sensitivity

Sensitivity of the LAMP assay was compared across a range of 10^5 - 10^1 copies per reaction (Table 4.2), using total DNA from an *X. fastidiosa* positive *V. vinifera* sample, diluted in clean *Vitis vinifera* extract. Copy number was calculated by qPCR (Francis *et al.* 2006) against a cloned DNA standard series; LAMP sensitivity was also assessed over a time-series of 60 through 90 minutes (Table 4.2). All samples were tested in duplicate, and the results tabulated for comparison.

4.2. RESULTS

The LAMP assay designed for amplification of *Xylella fastidiosa* was developed using published protocols (Varga and James 2006; Tomlinson *et al.* 2007) as reference points. It can be seen from Figure 1, that successful lamp amplification produces a ‘ladder-like’ pattern of amplified products when examined by gel electrophoresis, with the major amplicon of the expected size (~190bp) being produced at high concentration. Significant amounts of small amplification products (~100bp) and primer are also readily observed.

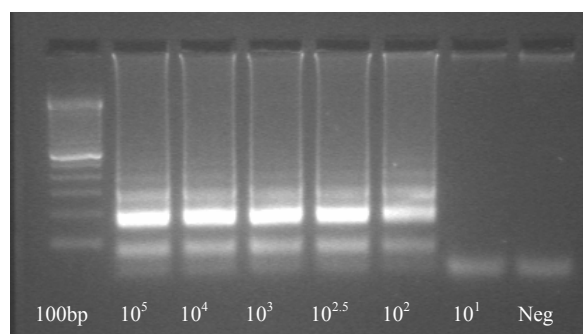


Figure 4.1: A dilution series of *Xylella fastidiosa* total DNA diluted in water amplified by LAMP using primers targeting the *rimM* gene. Initial copy number per μl of loaded total DNA is given.

As the reaction protocol of Varga & James (2006) proved to be effective during initial testing with a small number of samples, only minor optimisation was conducted. It was found that Betaine concentration was critical, and reduction below 0.8M prevented successful amplification, while varying reaction temperature between 60 to 65 degrees, had no noticeable effect. However, testing of the *rimM* and other designed primer sets indicated that HPLC purification of primers was required for rapid and successful amplification.

4.2.1 LAMP specificity

The *X. fastidiosa-rimM* LAMP assay designed in this study was tested against DNA obtained from a range of *X. fastidiosa* isolates, healthy plant tissue and other bacterial species (Table 4.1). The LAMP assay was found to amplify all *X. fastidiosa* isolates from various hosts including all members of the major four subspecies, *X. fastidiosa* subsp. *fastidiosa*, *multiplex*, *sandyi* and *pauca*. No amplification was observed for the causal agent of Pear leaf scorch from Taiwan, which is classified as *X. fastidiosa*, yet is genetically distinct. No cross-reaction or non-specific amplification was observed with DNA from closely related bacterial species within the genus *Xanthomonas*, or from *Spiroplasma citri* which is a bacterium that may found in the same host as *X. fastidiosa*. There was also no cross-reaction with common host species: *Vitis vinifera*, *V. rotundifolia*, *Prunus persica* or *Citrus latifolia*.

The specificity of the LAMP assay was compared with the standard PCR and real-time PCR assays (Table 4.1). The results showed that the standard PCR was unable to amplify three and four samples from subspecies *fastidiosa* and *multiplex* respectively. It was however, the only method tested that successfully amplified DNA from the Pear leaf scorch *X. fastidiosa* sample. The standard PCR method cross-reacted with one of the *Xanthomonas* samples, although this result was not confirmed by sequencing. The real-time PCR method was generally more effective than the standard PCR, amplifying all but two of the subspecies *multiplex* samples, and giving no observable cross-reaction with non-target species.

Table 4.1 Samples of *X. fastidiosa* used for specificity testing in this study. Specificity results for the three methods applied, LAMP, PCR and qPCR, are given as follows: '+' successful amplification, '-' negative or no amplification, and '?' ambiguous or non-specific amplification.

| Species | Host | Country of Origin | Sample Type | Source | LAMP | qPCR and PCR | | |
|---|--|------------------------|-------------|-------------------------|-------------|------------------------------|--------------------------------|---|
| | | | | | <i>rimM</i> | Francis <i>et al.</i> (2006) | Minsavage <i>et al.</i> (1994) | |
| <i>X. fastidiosa</i> subsp. <i>fastidiosa</i> | <i>Vitis vinifera</i> | Unknown | Culture | DSMZ cat. 10026 | + | + | + | |
| | <i>Vitis vinifera</i> | Unknown | Culture | Sediag cat. Xylf-LPC | + | + | + | |
| | <i>Vitis vinifera</i> | Unknown | Culture | Loewe cat. 07119pc | + | + | + | |
| | <i>Vitis vinifera</i> | USA | Culture | L. Nunney, UC Riverside | + | + | - | |
| | <i>Vitis vinifera</i> | USA | Culture | L. Nunney, UC Riverside | + | + | + | |
| | <i>Vitis vinifera</i> | USA | Tissue | R. Almeida, UC Berkley | + | + | - | |
| | <i>Vitis vinifera</i> 'Merlot' | USA | Tissue | R. Almeida, UC Berkley | + | + | - | |
| | <i>Prunus dulcis</i> | USA | Culture | L. Nunney, UC Riverside | + | + | + | |
| | <i>Prunus dulcis</i> | USA | Culture | L. Nunney, UC Riverside | + | + | + | |
| | <i>Prunus dulcis</i> | USA | Culture | L. Nunney, UC Riverside | + | + | + | |
| | <i>Vitis rotundifolia</i> | USA | Tissue | C. Chang, U Georgia | + | + | - | |
| | <i>Graphocephala atropunctata</i> | USA | Tissue | R. Almeida, UC Berkley | + | + | + | |
| | <i>X. fastidiosa</i> subsp. <i>multiplex</i> | <i>Prunus salicina</i> | USA | Culture | ICMP-8375 | + | + | + |
| | | <i>Prunus dulcis</i> | USA | Culture | ICMP-6575 | + | + | + |
| <i>Prunus dulcis</i> | | USA | Culture | L. Nunney, UC Riverside | + | + | + | |
| <i>Quercus laevis</i> | | USA | Culture | L. Nunney, UC Riverside | + | + | - | |
| <i>Quercus rubra</i> | | USA | Culture | L. Nunney, UC Riverside | + | - | - | |
| <i>Quercus rubra</i> | | USA | Tissue | C. Chang, U Georgia | + | + | - | |
| <i>Liquidambar styraciflua</i> | | USA | Culture | L. Nunney, UC Riverside | + | - | - | |
| <i>X. fastidiosa</i> subsp. <i>sandyi</i> | <i>Nerium oleander</i> | USA | Culture | L. Nunney, UC Riverside | + | + | + | |
| | <i>Nerium oleander</i> | USA | Culture | L. Nunney, UC Riverside | + | + | + | |
| | <i>Nerium oleander</i> | USA | Culture | L. Nunney, UC Riverside | + | + | + | |

| | | | | | | | |
|--|---------------------------|--------|---------|-------------------------------|---|---|---|
| <i>X. fastidiosa</i> subsp. <i>pauca</i> | <i>Citrus</i> sp. | Brazil | Culture | H. Coletta Filho, CDC Brasil. | + | + | + |
| | <i>Citrus</i> sp. | Brazil | Tissue | H. Coletta Filho, CDC Brasil. | + | + | + |
| Unknown | <i>Pyrus</i> sp. | Taiwan | Tissue | C. Su | - | - | + |
| <i>Xanthomonas axonopodis</i> | <i>Citrus latifolia</i> | Brazil | Culture | ICMP 14285 | - | - | ? |
| <i>Xanthomonas campestris</i> pv. <i>citri</i> | <i>Citrus paradisi</i> | USA | Culture | ICMP 10012 | - | - | - |
| | <i>Citrus limon</i> | NZ | Culture | ICMP-24 | - | - | - |
| <i>Spiroplasma citri</i> | <i>Citrus</i> sp. | USA | Tissue | R. Yokomi, USDA | - | - | - |
| Healthy Host Species | <i>Vitis vinifera</i> | NZ | Tissue | MAF Collection | - | - | - |
| | <i>Vitis rotundifolia</i> | USA | Tissue | MAF Collection | - | - | - |
| | <i>Prunus persica</i> | NZ | Tissue | MAF Collection | - | - | - |
| | <i>Citrus latifolia</i> | NZ | Tissue | MAF Collection | - | - | - |

4.2.2 LAMP sensitivity

The sensitivity of the LAMP assay was examined using a serial dilution of *X. fastidiosa* total DNA, diluted in healthy *Vitis vinifera* DNA across a range of 10^4 through to 10^1 copies per reaction (Table 4.2). It was found that sensitivity increased with incubation time from 60 to 75 minutes, but not over 75 minutes, with a limit of detection of 200-250 copies per reaction. Amplification of 100 copies per reaction was observed, but was not reproducible. This can be compared to standard PCR, with a successful detection limit of 500 copies, and real-time qPCR with a detection limit of 10 copies, although this level was not accurately quantifiable and subject to inconsistencies in accurately pipetting such a low copy number concentration. The conventional PCR sensitivity is also misleading as there are three copies of the target gene, as opposed to one copy for the LAMP and real-time assay gene targets.

Table 4.2: Sensitivity of the *X. fastidiosa* specific LAMP reaction over time against a serial dilution of *Xylella fastidiosa* compared to standard and real-time qPCR assays.

| Concentration of <i>X. fastidiosa</i> template as determined by qPCR | Test | | | | |
|--|------------------|---------|---------|------------------------------|--------------------------------|
| | <i>rimM</i> LAMP | | | qPCR and PCR | |
| | 60 Mins | 75 Mins | 90 Mins | Francis <i>et al.</i> (2006) | Minsavage <i>et al.</i> (1994) |
| 10000 copies per reaction | + | + | + | + | + |
| 1000 copies per reaction | + | + | + | + | + |
| 500 copies per reaction | + | + | + | + | + |
| 250 copies per reaction | - | + | + | + | + (Faint) |
| 100 copies per reaction | - | +/- | +/- | + | - |
| 10 copies per reaction | - | - | - | + | - |
| Negative | - | - | - | - | - |

4.2.3 LAMP reliability

The LAMP reaction was found to be reliable for samples containing a concentration of greater than 250 target copies per reaction; lower concentrations of target DNA are subject to operator loading error in that the amount of DNA can not be consistently pipetted. The same issue applies for low copy numbers of target DNA when using real-time PCR. As conventional PCR is less sensitive than both the LAMP and real-time assays, detection cut-off is generally above a copy number where loading error becomes an issue. The detection limit of an assay can be affected by a number of things including the presence of inhibitory

compounds such as phenolics and polysaccharides, secondary structures of primers and target DNA, the type of enzyme used in the reaction, and the sensitivity of the end point detection method itself. It may be stated that the LAMP reaction functions effectively and consistently at target concentrations of 250 copies of the target gene or greater, and is able to detect representative isolates from all major subspecies of *X. fastidiosa*; differences in sample type and extraction method applied did not produce noticeable differences in reaction efficacy.

5. Evaluation of DNA Extraction Methods

Deliverable 8: Evaluate DNA extractions from plants and insect vectors which can be used in conjunction with the LAMP assay outside the laboratory.

5.1. MATERIALS AND METHODS

5.1.1 Samples

Lyophilised *X. fastidiosa*-infected tissue samples of *Vitis vinifera*, *V. rotundifolia* and *Quercus rubra* leaves, and infected blue-green sharpshooters (*Graphocephala atropunctata*) were obtained from Dr. R. Almeida (University of California, Berkley, CA, USA) and C. Chang (University of Georgia, USA).

5.1.2 DNA extraction using standard laboratory methods

Both lyophilised plant tissue and infected blue-green sharpshooters (BGSS), were initially processed by grinding to a fine powder in liquid nitrogen followed by DNA extraction using either the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) as described by (Huang *et al.* 2006), or, for plant tissue only, the more rapid magnetic-bead based KingFisher mL system (Thermo-Fisher Scientific, Waltham MA, USA) as described by the manufacturer. The KingFisher mL system, DNA was also used to extract DNA from plant tissue which had not been initially ground in liquid nitrogen. These methods were used as a reference for comparing field based techniques.

5.1.3 Alternative field-based DNA extraction methods for plants

Two alternative DNA extraction methods that could be used in the field with minimal specialised equipment were also trialled: the Extract-N-Amp Kit (Sigma-Aldrich), which uses chemical and heat disruption, as per the manufacturer's protocol, and the Bio-Nobile 8-M PickPen[®] (Bio-Nobile, Turku, Finland) using Invimag Plant DNA KFmL Mini Kit reagents (Invitek). For the latter, samples (200 mg) of infected lyophilised petiole and leaf midrib tissue were homogenised in 2 ml of lysis buffer P (Invitek) in sample extraction bags (BioReba, Basel, Switzerland) using a hand-roller. DNA extraction was then performed in a Nunc 96 DeepWell plate (Thermo-Fisher Scientific, Waltham, MA, USA) using the PickPen[®] to manipulate the magnetic beads. Briefly, 420 µl of homogenised plant sap was added to the first well with 20 µl of magnetic beads and 200 µl binding buffer and mixed using the PickPen[®] for three minutes. Beads were collected and transferred into 800 µl of wash buffer 1 for two minutes, followed by two washes of two minutes with 800 µl of wash buffer 2. DNA was eluted for three minutes in 100 µl of nuclease-free H₂O. DNA was stored at -80°C prior to use.

5.1.4 Alternative field-based DNA extraction methods for invertebrates

DNA from BGSS were extracted using two insect specific methods, first using the heat and chemical based PrepGem invertebrate kit (Zygem Ltd., Hamilton, New Zealand) as per the manufacturer's instructions, and second using Chelex resin as described (Walsh *et al.* 1991). Due to the limited amount of BGSS available, no extraction was made using the PickPen or Invimag/KingFisher systems.

5.1.5 Testing of extraction methods

Samples extracted using all methods were tested using three protocols: the *rimM*-based LAMP assay designed for this study and the real-time PCR assay of Francis *et al.* (2006) to test for *X. fastidiosa* extraction, and the cytochrome oxidase (COX) assay of Weller *et al.* (2000) to indicate PCR competency of each DNA extraction.

5.2. RESULTS

Several alternative methods for the extraction of total nucleic acids from plant and invertebrate tissue were trialled and compared to the standard DNeasy column-based method. DNA extracted from infected lyophilised tissue of *V. vinifera*, *V. rotundifolia* and *Q. rubra*, and BGSS insects was tested using the LAMP and the *rimM* real-time PCR assays developed in this study. PCR competency was checked using a real-time internal control assay for the cytochrome oxidase (COX) gene (Weller *et al.* 2000); results are presented in Table 5.1.

Table 5.1: Comparison of laboratory and field-based extraction methods to the DNeasy method, assessed using *X. fastidiosa rimM* LAMP and real-time PCR assays and a COX internal control assay to show PCR competency. +: positive; -: negative.

| Tissue | Extraction Method | Test | | |
|--|------------------------|------------------|---------------|-----|
| | | <i>rimM</i> LAMP | Real-time PCR | COX |
| <i>Vitis vinifera</i> cv. Cabernet Sauvignon | DNeasy | + | + | + |
| | KFmL w/ Liq. N2 | - | - | + |
| | KFmL | - | - | - |
| | PickPen w/ Invimag Kit | + | + | + |
| | Sigma Extract n' Amp | - | - | - |
| <i>Vitis rotundifolia</i> | DNeasy | + | + | + |
| | KFmL w/ Liq. N2 | + | - | + |
| | KFmL | - | + | - |
| | PickPen w/ Invimag Kit | + | + | + |
| | Sigma Extract n' Amp | - | - | - |
| <i>Quercus rubra</i> | DNeasy | + | + | + |
| | KFmL w/ Liq. N2 | + | + | + |
| | KFmL | + | + | + |
| | PickPen w/ Invimag Kit | + | + | + |
| | Sigma Extract n' Amp | - | - | - |
| <i>Graphocephala atropunctata</i> | DNeasy | + | + | + |
| | PrepGEM | + | + | + |
| | Chelex | + | - | + |

It can be seen that neither the COX internal control or *X. fastidiosa* were amplified from DNA extracted using the Extract-N-Amp method. In addition, amplification from DNA extracted using the Kingfisher method and without tissue disruption using liquid nitrogen was largely sample dependant with extractions from *Vitis* tissue giving inconsistent results. This is probably largely due to the presence of PCR inhibitors in this tissue type. In contrast, DNA extracted from all host types using the DNeasy methods and the field-based PickPen[®]/Invimag protocol produced consistent amplification of both internal control and *X. fastidiosa*. However, DNA amplified on average, 6.45 cycles earlier for COX and 0.40 cycles earlier for *X. fastidiosa* using the DNeasy compared to the PickPen[®] method. These results, suggest that although field extraction by PickPen[®] is possible, it may not be as effective as the column-based technique for recovering low titres of *X. fastidiosa* DNA. As liquid nitrogen is not feasible for use in the field it was not tested using the Extract n' Amp or PickPen assays.

Similar results were obtained for BGSS DNA extracted using all methods as both LAMP and COX assays amplified successfully. However, *X. fastidiosa* was not amplified by real-time PCR when extracted using the chelex resin based extraction. Given the sensitivity of real-time PCR to inhibitors, this is not surprising as chelex is a crude means of extraction.

6. LAMP endpoint detection methods for field-based testing

Evaluate endpoint detection (gel-free) methods of confirming successful LAMP amplification that are applicable for field testing (Extra work completed, not in milestones).

6.1. METHODS

A total of three methods: hydroxynaphthol blue (HNB) (Sigma-Aldrich, St. Louis, MO, USA), PicoGreen (Invitrogen, Carlsbad, CA, USA), and lateral-flow devices with specific FITC and Biotin-labelled primers were assessed and compared to gel electrophoresis.

6.1.1 Hydroxynaphthol blue

Hydroxynaphthol Blue was added to the optimised 25 µl rimM LAMP reaction master-mix at a final concentration of 150 µM, as recommended by Goto *et al.* (2009), prior to incubation. Successful amplification was indicated by a colour change from purple to blue (Figure 6.1). Reactions were observed at 45, 60 and 75 minutes and confirmation of amplification was performed by gel electrophoresis.

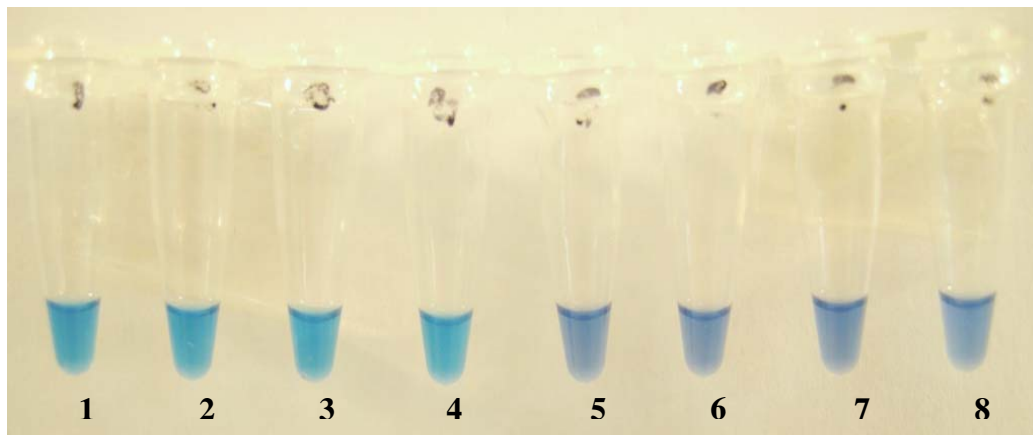


Figure 6.1: An example of positive (blue, tubes 1-4) and negative (purple, tubes 5-8) loop-mediated isothermal amplification using hydroxynaphthol dye as an indicator.

6.1.2 PicoGreen

After performing LAMP amplification for 45, 60 and 75 minutes using the optimised *rimM* protocol, as described in section four of this report, 2 µl of PicoGreen (Invitrogen) was added to each tube and observed visually. A colour change from orange to yellow-green indicated the presence of amplified DNA.

6.1.3 Lateral flow devices

LAMP amplification for LFD testing was performed using the optimised *rimM* protocol as described in section four of this report, with the substitution of conventional unlabelled LF and LB primers with fluorescein isothiocyanate (FITC) labelled LF and Biotin labelled LB primers. After incubation for 45, 60 or 75 minutes a 1 µl aliquot from each reaction was diluted 1:500 in LFD Buffer C (FERA, Sand Hutton, UK) and placed onto lateral flow devices. The appearance of blue lines in both sample and control lines indicated positive amplification.

6.2. RESULTS

The sensitivity of the three endpoint methods tested, HNB, PicoGreen and LFD (Table 6.1) were found to be similar, with consistent detection of 250 copies per reaction at 75 minutes. The LFDs were, however, much more sensitive at 45 and 60 minutes, while HNB and PicoGreen results were comparable.

A comparison of the colorimetric results for both HNB and PicoGreen to their respective gel electrophoresis results suggested that both were largely consistent, although four false positives were observed using PicoGreen, and one false negative for HNB; given the subjective nature of determining colour change for PicoGreen in particular, this is to be expected. Some contamination was observed using PicoGreen, which is a consequence of the need to open the tubes to add the intercalating dye, therefore this cannot be recommended as a reliable method. No contamination was observed using HNB.

The lateral flow devices by contract showed high sensitivity over all measurements, although did produce one false positive. Given the sensitivity of the LFD method, cross-contamination was a significant issue.

Table 6.1: Comparison of endpoint detection methods for screening of LAMP amplification reactions in the field. '+': positive; '-': negative, and '?': faint or indeterminate results.

| A) Hydroxynaphthol Blue | | | | | | | |
|-------------------------|--------------------------|----------------------|---------|---------|--------------------------|---------|---------|
| Type | Host | Hydroxynaphthol Blue | | | Hydroxynaphthol Blue Gel | | |
| | | 45 mins | 60 mins | 75 mins | 45 mins | 60 mins | 75 mins |
| Dilution | 1000 copies per reaction | ++- | +++ | +++ | N/A | N/A | +++ |
| Sensitivity | 500 copies per reaction | --- | ++- | +++ | N/A | N/A | +++ |
| <i>Vitis</i> | 250 copies per reaction | --- | ?-- | +++ | N/A | N/A | +++ |
| <i>vinifera</i> | 125 copies per reaction | --- | --- | +- | N/A | N/A | ++- |
| 'Merlot' | Water | --- | --- | --- | N/A | N/A | --- |
| +ve | | --- | --- | --- | N/A | N/A | --- |

| B) PicoGreen | | | | | | | |
|-----------------|--------------------------|-----------|---------|---------|---------------|---------|---------|
| Type | Host | PicoGreen | | | PicoGreen Gel | | |
| | | 45 mins | 60 mins | 75 mins | 45 mins | 60 mins | 75 mins |
| Dilution | 1000 copies per reaction | +- | +++ | +++ | +++ | +++ | +++ |
| Sensitivity | 500 copies per reaction | --- | +++ | +++ | ?-- | +++ | +++ |
| <i>Vitis</i> | 250 copies per reaction | --- | +- | +++ | ?-- | +- | +++ |
| <i>vinifera</i> | 125 copies per reaction | --- | +- | +++ | --- | +- | +++ |
| 'Merlot' | Water | --- | --- | +- | --- | --- | +- |
| +ve | | --- | --- | +- | --- | --- | +- |

| C) Lateral Flow Device | | | | | | | |
|------------------------|--------------------------|---------|---------|---------|---------|---------|---------|
| Type | Host | LFD | | | LFD GEL | | |
| | | 45 mins | 60 mins | 75 mins | 45 mins | 60 mins | 75 mins |
| Dilution | 1000 copies per reaction | ++ | ++ | ++ | ++ | ++ | ++ |
| Sensitivity | 500 copies per reaction | ++ | ++ | ++ | ++ | ++ | ++ |
| <i>Vitis</i> | 250 copies per reaction | ++ | ++ | ++ | ++ | ++ | ++ |
| <i>vinifera</i> | 125 copies per reaction | ++ | ?? | +- | ++ | ++ | +- |
| 'Merlot' | Water | -- | -- | -+ | -- | -- | -+ |
| +ve | | -- | -- | -+ | -- | -- | -+ |

These data suggest that the use of hydroxynaphthol blue is the most acceptable solution for field- based screening of LAMP amplification products as it is both cost effective and does

not require opening the tubes after the reaction is complete, unlike the PicoGreen and LFD methods.

7. Technology transfer

Deliverable 9. Transfer technology to MAF, including development of standard operating procedures.

Work instructions and a worksheet to allow staff to record sample information when performing LAMP reactions have been developed and are stored in ECMS at the following links.

IDC Work Instructions Loop Mediated Isothermal Amplification of *Xylella fastidiosa*.doc

<http://fcs.maf.govt.nz/webtop/drl/objectId/090101b38059e09c>

FCS Folder Location: <http://fcs.maf.govt.nz/webtop/drl/objectId/0b0101b38018d6ef>

IDC Worksheet LAMP Xylella.xls

<http://fcs.maf.govt.nz/webtop/drl/objectId/090101b38059e08e>

FCS Folder Location: <http://fcs.maf.govt.nz/webtop/drl/objectId/0b0101b380132983>

8. Final Summary

From a quarantine perspective, the ability to exclude important regulated plant pathogens or undertake surveillance depends on rapid and reliable methods of detection. These methods must be easily transferable between laboratories and if possible be suitable for use in the field. The objective of this work was to develop such a diagnostic method for *X. fastidiosa*. LAMP (Notomi *et al.* 2000; Nagamine *et al.* 2002) seemed an appropriate method, as it has been used to amplify and detect plant pathogenic bacteria, fungi and viruses (Fukuta *et al.* 2003; Okuda *et al.* 2005; Varga and James 2006; Kubota *et al.* 2008; Tomlinson *et al.* 2010), as well as human and animal pathogens (Iwamoto *et al.* 2003; Savan *et al.* 2004; Song *et al.* 2005; Dukes *et al.* 2006; En *et al.* 2008). The reaction can potentially be performed in the field as minimal equipment is needed to run the reaction and positive reactions can be identified visually using colorimetric dyes (Goto *et al.*, 2009).

The LAMP assay is highly specific and shows greater sensitivity than conventional PCR. However, it is not as sensitive as real-time PCR which is consistent amongst LAMP assays designed for plant bacterial and viral pathogens (Fukuta *et al.* 2003; Okuda *et al.* 2005; Varga and James 2006; Kubota *et al.* 2008; Tomlinson and Boonham 2008). The level of sensitivity (~500 copies of template per reaction) obtained by LAMP is acceptable for first-instance screening, although with the caveat that samples of marginal titre or of poor DNA quality may be missed. It was also noted during assay development using *X. fastidiosa* DNA diluted in water versus dilution in healthy grapevine DNA, that the LAMP assay was less sensitive to inhibition than conventional and real-time PCR. The LAMP assay also is rapid, being able to detect *X. fastidiosa* extracted from infected tissue using a simple magnetic-bead based method in approximately one hour, similar to that described previously for real-time PCR (Schaad *et al.* 2002; Tomlinson *et al.* 2007).

A consideration for field-based detection with any assay is DNA extraction. Standard laboratory-based methods are not easily applied in the field due to the need for specialised equipment, and many of the field-based methods proposed are specific to each host-pathogen system (Kuske *et al.* 1998; Tomlinson *et al.* 2005). Being xylem-limited, *X. fastidiosa* presents a particular difficulty for field-based extraction since physical disruption of the tissue or extraction of sap is required (Schaad *et al.* 2002). In this study the Extract-N-Amp method which relies on thermal and chemical degradation failed to extract viable DNA, whereas homogenisation with a hand-roller and DNA extraction using magnetic beads with a hand-held device (PickPen[®]) was sufficient to extract *X. fastidiosa* DNA from lyophilised samples. Only lyophilised tissue was available for this study and therefore a comparison could not be made with the sap extraction protocol of Schaad *et al.* (2002). Using the PickPen[®] extraction, the titre of extracted *X. fastidiosa* DNA was on average 100-fold lower than in samples extracted using the Qiagen DNeasy system, as evidenced by lower Ct values, although Ct values for the internal control were not markedly different between the extraction types. Such a loss of sensitivity may cause false negative results for samples of marginal titre. Further extractions using fresh tissue may give a better indication of the performance of the PickPen[®] method and the use of additives to reduce the presence of inhibitors could be investigated. For invertebrate vectors of *X. fastidiosa* the PrepGem system, which uses chemical and heat degradation, was more effective than chelex. As magnetic bead systems such as the PickPen were not tested no comparison can be made, although based on experience it may be suggested that the PickPen would be suitable for invertebrate DNA extraction.

The result of a LAMP reaction may be assessed colorimetric hydroxynaphthol blue dye (Goto *et al.* 2009), which unlike fluorescent intercalating dyes such as SYBR or PicoGreen

(Tomlinson *et al.* 2007) or lateral flow devices with labelled primers (Tomlinson *et al.* 2010), does not require opening the tubes post-reaction. This reduces the chance of cross-over contamination caused by the high titre of the LAMP amplicon (Tomita *et al.* 2008). During this study, HNB required at least 500 copies of target template to trigger colour change within an hour, so there is a risk that very low titres of *X. fastidiosa* may not be detected if the assay is only run for an hour. However, when the assay was run for 75 minutes, all tissue samples tested were successfully amplified.

Finally, the cost the LAMP assay may be considered. The LAMP assay, with its requirement for specialised enzymes and reagents (especially dNTP usage and the need for HPLC-purified loop primers) costs approximately five times more for consumables than conventional or real-time PCR. However, this does not include the cost of specialised equipment such as real-time thermocyclers. The cost of LAMP may be a limitation for large-scale surveys, yet despite this method offers several advantages; there would be a huge saving in time if reactions are carried out in the field, it would reduce the movement of infected tissue across the country for laboratory testing, and non-scientific staff could be trained to use the method due to its simplicity.

In conclusion, it may be stated the LAMP *rimM* assay (the optimised protocol is described in Appendix 1) has a high level of specificity for the detection and diagnosis of the major subspecies of *X. fastidiosa*. Provided that care is taken to avoid contamination by running LAMP as a closed-tube reaction (Tomita *et al.* 2008) with HNB as a reporter dye, LAMP has the potential to be used in the field. Initial work comparing extraction methods suggest that using the PickPen magnetic bead extraction procedure for plants and PrepGem for insects, suitable extraction techniques can be used alongside LAMP *in situ*. The method described here is readily transferable to other laboratories due to the fact that expensive specialised equipment is not required. It is estimated that a reasonable number of plants and insects samples (>20) could be processed/screened within two hours in the field.

9. Appendix 1: LAMP Protocol

| Reagents | Volume per reaction (µl) |
|---|--------------------------|
| 10x ThermoPol Reaction Buffer | 2.50 |
| 50mM MgSO ₄ (6 mM - to 8 mM final) | 3.00 |
| 5M Betaine (0.8 M final) | 4.00 |
| 10mM dNTPs (1.4 mM final) | 3.50 |
| 5µM F3 Primer (0.2 µM final) | 1.00 |
| 5µM B3 Primer (0.2 µM final) | 1.00 |
| 40µM FIP Primer (1.6 µM final) | 1.00 |
| 40µM BIP Primer (1.6 µM final) | 1.00 |
| 20µM F-loop Primer (0.8 µM final) | 1.00 |
| 20µM B-loop Primer (0.8 µM final) | 1.00 |
| <i>Bst</i> DNA Polymerase 8 U/µl | 1.00 |
| 10mM Hydroxynaphthol Blue (150 µM final) | 0.30 |
| DNA Template | 2.00 |
| Sterile MilliQ water | 2.70 |
| Total Volume | 25.00 |

Incubate at 65 °C for 60-75 minutes, examine results visually or perform gel electrophoresis.

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