Development of LAMP and Real-Time PCR Methods for the Rapid Detection of *Xylella fastidiosa* for Quarantine and Field Applications

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ABSTRACT

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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) and real-time polymerase chain reaction (PCR) assay were developed to the *rimM* gene of *X. fastidiosa* and evaluated for specificity and sensitivity. Both assays were more robust than existing published assays for detection of *X. fastidiosa* when screened against 20 isolates representing the four major subgroups of the bacterium from a range of host species. No cross-reaction was observed with DNA from healthy hosts or other bacterial species. The LAMP and real-time assays could detect 250 and 10 copies of the *rimM* gene, respectively, and real-time sensitivity was comparable with an existing published real-time PCR assay. Hydroxynapthol blue was evaluated as an endpoint detection method for LAMP. When at least 500 copies of target template were present, there was a noticeable color change indicating the presence of the bacterium. Techniques suitable for DNA extraction from plant tissue in situ were compared with a standard silica-column-based laboratory extraction method. A portable PickPen and magnetic bead system could be used to successfully extract DNA from infected tissue and could be used in conjunction with LAMP in the field.

Additional keywords: diagnostics.

Xylella fastidiosa (39) is a bacterial plant pathogen that causes several economically important diseases, including Pierce's disease of grapevine, citrus veinal chlorosis, almond leaf scorch, phony peach, and leaf scorch on a range of ornamental plants and shade trees (10–12). *X. fastidiosa* is a regulated organism in many parts of the world. Leafhoppers of the subfamily *Cicadellinae* (*Hemiptera: Cicadellidae*) and spittle bugs or frog hoppers of the family *Cercopidae* (*Hemiptera*) are the most common known vectors (27). The distribution of *X. fastidiosa* is generally limited to the Americas (27), with two exceptions, in *Vitis vinifera* in Kosovo (1) and pear in Taiwan (18). It is thought that *X. fastidiosa* is sensitive to low temperatures, which has restricted its movement into regions with temperate climates and, in particular, cold winters (27). However, many colder parts of the world do possess one or more vector species, such as the spittlebug (*Philaenus spumarius*); therefore, the potential does exist for *X. fastidiosa* to spread into such areas should cold-tolerant strains, such as almond leaf scorch, become established (27). From a quarantine perspective, rapid detection and diagnosis is the key feature of any exclusion strategy.

Current assays for *X. fastidiosa* diagnosis include bacterial cell culture, conventional polymerase chain reaction (PCR) (12,13, 20,26,28), and real-time PCR (7,29). Although many of these methods have been used routinely in the laboratory, most of these methods are not easily transferable to the field. In addition, the PCR assay of Minsavage et al. (20) was developed over 15 years ago when there was little DNA sequence of *X. fastidiosa* available. This assay is commonly used for quarantine screening and, therefore, it is particularly important to verify that it detects all isolates of the bacterium reliably. In view of these factors, alternative methods of detection were considered.

One method that has been recently adopted for plant pathogen diagnostics is loop-mediated isothermal amplification (LAMP). Because the LAMP reaction is isothermal, it can be performed in a heat block or water bath, thereby removing the need for specialized equipment. In addition, positive amplification can be observed by colorimetric or fluorescent dyes (9,33), removing the need to run gels. Both of these factors contribute to transferability to the field.

Here, we present the development and evaluation of a LAMP assay for *X. fastidiosa* to improve diagnostic capability by enabling surveillance activities, improving response times during incursions, and allowing testing of imported commodities at the border. During the development of the LAMP assay, the potential arose to develop an alternative real-time TaqMan (Applied Biosystems, Foster City, CA) PCR based on detection of the same region used for the LAMP primer design. The new TaqMan (Applied Biosystems) real-time assay was also evaluated alongside the LAMP method.

MATERIALS AND METHODS

Samples. *X. fastidiosa* cultures were obtained from commercial (DSMZ, Mannheim, Germany) and academic (Landcare Research, Auckland, New Zealand) sources. Freeze-dried *X. fastidiosa*-infected samples of *V. vinifera*, *V. rotundifolia*, and *Quercus rubra* leaves and infected blue-green sharpshooters (*Graphocephala atropunctata*) were obtained from Dr. R. Almeida (University of California, Berkley) and C. Chang (University of Georgia, Griffin). DNA samples of *X. fastidiosa*, extracted from a range of host species, were obtained either on FTA cards (Whatman Inc., Florham Park, NJ) or lyophilized, from Dr. L. Nunney (University of California, Riverside), Dr. C. Su (Taiwan Agricul-

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tural 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). Finally, DNA extracts of healthy host-plant species and nontarget bacterial species were obtained from the MAF Biosecurity New Zealand nucleic acid collection.

Sample DNA extraction. Plant samples (200 mg of leaf midrib and petiole) and whole insects were ground to a fine powder in liquid nitrogen prior to extraction using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) as per published protocols (13). Samples on FTA cards (Whatman Inc.) were eluted using the Sigma Extract-N-Amp Kit (Sigma-Aldrich, St. Louis) using the manufacturer's protocol. Lyophilized cell cultures were disrupted with a Roche MagNA Lyser instrument (Roche Applied Science, Auckland, New Zealand), then extracted using DNeasy Plant Mini Kit (Qiagen) as above.

Testing alternative DNA extraction methods for field use. Two alternative DNA extraction methods that could potentially be used in the field with minimal specialized equipment were tested: the Extract-N-Amp Kit (Sigma-Aldrich) using the manufacturer's protocol and the Bio-Nobile 8-M PickPen (Bio-Nobile, Turku, Finland) using Invimag Plant DNA KFmL Mini Kit reagents (Invitek, Berlin, Germany). Samples (200 mg) of infected lyophilized petiole and leaf midrib tissue were homogenized 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) using the PickPen to manipulate the magnetic beads. Briefly, 420 µl of homogenized plant sap was added to the first well with 20 µl of magnetic beads and 200 µl of binding buffer and mixed using the PickPen (Bio-Nobile) for 3 min. Beads were collected and transferred into 800 µl of wash buffer 1 for 2 min, followed by two washes of 2 min with 800 µl of wash buffer 2. DNA was eluted for 3 min in 100 µl of nuclease-free H_2O. DNA was stored at –80°C prior to use.

Gene target selection and primer design. Using the work of Doddapaneni et al. (5) as a starting point, potential gene targets within the *X. fastidiosa* genome were assessed on the basis of sequence conservation between isolates with an arbitrary threshold of >98% nucleotide identity, and significant sequence difference from related species in the family *Xanthomonadaceae*. In total, four candidate genes were selected for primer design from the data of Doddapaneni et al. (5) and an alignment of the extant genomes 9a5C, Temecula, M12, and M23 (National Center for Biotechnology Information GenBank accession numbers NC_002488, NC_004556, NC_010513, and NC_010577, respectively). These genes were annotated as per the genome of the 9a5c isolate: disulfide isomerase (XF_1834), the 16S rRNA processing protein *rimM* (XF_0108), a HicB-related protein (XF_1668), and a cell division protein (XF_0095). Two additional regions, citrate synthase *gltA* (XF_1535) (2) and a hypothetical protein (7) used in published assays for *X. fastidiosa* detection and diagnosis, were also selected.

All potential target regions were examined for primer design using the alignment of the *X. fastidiosa* genomes described above and the online PrimerExplorer V4 software (Eiken Chemical Co., Tokyo) with default program parameters. Viable primer sets were generated for three targets—disulfide isomerase, *rimM,* and *gltA*—initial testing of which (data not shown) led to the adoption of the *rimM* primer set (Table 1) for further development and testing. The two inner primers designed, XF-FIP and XF-BIP, were modified with a TTTT linker sequence between the sense and antisense sequences to ensure loop formation, and two complimentary loop primers, XF-LF and XF-LB, were designed to accelerate strand displacement and amplification (23).

Following the selection of the LAMP primers, a set of real-time PCR primers and the associated 6′-carboxyfluorescein/Black Hole Quencher-1-labeled (6′FAM/BHQ) TaqMan (Applied Biosystems) probe (Table 1) were also designed to the *rimM* open reading frame (ORF) using the online RealTimeDesign software (BioSearch Technologies, Novato, CA) with the default parameters.

Optimization of the rimM LAMP assay. The LAMP protocol was developed from the method described by Varga and James (37). To optimize the LAMP reaction, the concentrations of core reagents were tested as follows: $MgSO_4$ at 4 to 8 mM and betaine at 0.6 to 1 M. Trehalose was examined as an alternative to betaine (30) at a concentration of 0.2 to 1 M. Reaction temperatures of 62 to 65°C were tested, as were optimal reaction times of 45 to 90 min. The optimized *rimM* LAMP reaction was performed in a 25 µl reaction volume containing 1× ThermoPol buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton X-100, pH 8.8) (New England Biolabs, Beverly, MA) with additional $MgSO_4$ to a total final concentration of 8 mM, 0.8 M Betaine, 1.4 mM each dNTP, 0.2 µM outer (XF-F3/XF-B3) primers, 0.8 µM loop (XF-LF/XF-LB) primers, and 1.6 µM inner primers (XF-FIP/XF-BIP), with eight units of *Bst* DNA polymerase (New England Biolabs) and 2 µl of total DNA extract (of 100 to 300 ng of total DNA, depending on sample type). The reaction was incubated at 65°C for 60 min, followed by a 2 min enzyme inactivation step at 80°C in an ABI 9700 thermocycler (Applied Biosystems). Successful amplification of *X. fastidiosa* DNA was confirmed by agarose gel electrophoresis of a 15 µl aliquot of the LAMP reaction. Target specificity of the LAMP assay was confirmed by sequencing of the major amplification product using the XF-LF and XF-LB primers using the Sanger method; sequencing was performed by Ecogene (Auckland, New Zealand).

Evaluation of hydroxynapthol blue. The addition of hydroxynapthol blue (HNB) (Sigma-Aldrich) as a colorimetric means of indicating positive reactions (9) was assessed. HNB was added at

TABLE 1. Primers designed for the amplification of the partial *rimM* open reading frame of *Xylella fastidiosa* by loop-mediated isothermal amplification (LAMP)[a] and real-time polymerase chain reaction[b]

Assay, primer	Sequence (5′–3′)	Binding site
qRT-PCR		
XF-F	CACGGCTGGTAACGGAAGA	106,620–106,602
XF-R	GGGTTGCGTGGTGAAATCAAG	106,550–106,570
XF-P	TCGCATCCCGTGGCTCAGTCC	106,601–106,584
LAMP		
XF-F3	CCGTTGGAAAACAGATGGGA	106,884–106,865
XF-B3	GAGACTGGCAAGCGTTTGA	106,676–106,694
XF-FIP	ACCCCGACGAGTATTACTGGGTTTTTCGCTACCGAGAACCACAC	106,788–106,862
XF-BIP	GCGCTGCGTGGCACATAGATTTTTGCAACCTTTCCTGGCATCAA	106,773–106,695
XF-LF	TGCAAGTACACACCCTTGAAG	106,824–106,844
XF-LB	TTCCGTACCACAGATCGCT	106,753–106,735

[a] LAMP primer binding sites are given for the genome of isolate 9a5C (GenBank accession no. NC_002488).
[b] Quantitative reverse-transcription polymerase chain reaction.

a concentration of 150 μM as described by Goto et al. (9) in a final reaction volume of 25 μl. The rate of color change from purple to blue was assessed from 45 to 75 min at 15-min intervals at 65°C using a dilution series of *X. fastidiosa* DNA extracted from lyophilized cultured cells (DSMZ), of 1,000, 500, 250, 125, and 10 copies per reaction diluted in clean (uninfected) *V. vinifera* total DNA extract; concentration was calculated using an estimated genome size of 2.5 MB with the formula copies per microliter = (concentration in nanograms × 6.023 × 10^{23})/(genome length × 1 × 10^9 × 650).

Real-time PCR optimization. The *X. fastidiosa rimM* real-time PCR assay designed in this study was optimized for primer and TaqMan (Applied Biosystems) probe concentration, Mg^{2+} and thermocycling conditions using Invitrogen quantitative (q)PCR Supermix-UDG (Invitrogen, Carlsbad CA), and a Bio-Rad CFX-96 gradient real-time thermocycler (Bio-Rad Laboratories, Hercules, CA). Final optimized reaction conditions were as follows: real-time PCR reactions were done in 20-μl reaction volumes containing 10 μl of 2× qPCR Supermix-UDG (Invitrogen) with a final concentration of 4 mM $MgCl_2$, 300 nM *X. fastidiosa* sense (XF-F) and antisense (XF-R) primers, 100 nM 6′FAM/BHQ-1-labeled XF-P probe, bovine serum albumin (BSA) at 300 ng/μl (Sigma-Aldrich), and 2 μl of total DNA template. Optimal thermocycling conditions were as follows: 50°C for 2 min and 94°C for 2 min, then 40 cycles of 94°C for 10 s and 62°C for 40 s. All samples were amplified in triplicate. Threshold values were applied automatically by the CFX Manager V1.6 software (Bio-Rad Laboratories). Reaction efficiency was calculated using the dilution series described above for HNB evaluation, with the formula $E = 10^{(-1/\text{slope})}$.

Comparison to extant methods. The sensitivity, specificity, and reliability of the *rimM* LAMP and real-time PCR assays were compared with published *X. fastidiosa* conventional PCR (20) and real-time PCR (7) assays. DNA from 20 isolates representing the four major subgroups of *X. fastidiosa* and the phylogenetically distinct pear leaf scorch isolate was tested with all assays, and positive amplification was determined by either the presence of a band of the expected size for LAMP and conventional PCR or a crossing threshold (Ct) value of <38 cycles for the real-time PCR assays. In addition, infected blue-green sharpshooters were also tested to determine whether the designed assays would amplify *X. fastidiosa* from the vector; healthy sharpshooters could not be sourced. Finally, DNA was extracted from cultures and plant and insect tissue as described above. DNA from nontarget bacterial species and healthy plant hosts were tested to check for cross-reactivity (Table 2). The sensitivity of each assay was determined using the dilution series described earlier. Confirmation of the copy numbers of the dilution series and an estimation of target concentration in the *X. fastidiosa*-positive sample extracts were obtained by absolute real-time PCR using the assay of Francis et al. (7) against a cloned DNA standard. Real-time PCR and LAMP assays were performed in triplicate, while conventional PCR was performed in duplicate.

Conventional PCR reactions were done in 20-μl reaction volumes containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 250 nM forward and reverse primers, 200 nM dNTPs, 1 unit of Platinum *Taq* DNA polymerase (Invitrogen), and 2 μl of DNA template in an ABI 9700 thermocycler (Applied Biosystems). Cycling conditions consisted of 3 min at 94°C; followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s; with a final extension step of 5 min at 68°C. PCR products were visualized on a 1.5% agarose gel using a 15-μl aliquot of PCR reaction.

The real-time PCR assay of Francis et al. (7) was performed with a Bio-Rad CFX-96 thermocycler (Bio-Rad Laboratories), using a reaction volume of 20 μl containing 2× qPCR Supermix-UDG (Invitrogen) with a final concentration of 4.5 mM $MgCl_2$, 300 nM *X. fastidiosa* sense and antisense primers, 100 nM 6′FAM-labeled probe, BSA at 300 ng/μl, and 2 μl of DNA template. Thermocycling conditions were 50°C for 2 min and 94°C for 2 min, then 40 cycles of 94°C for 10 s and 60°C for 45 s. The optimized *rimM*-specific LAMP and real-time PCR assays were performed as described above.

RESULTS

Primer design. A conserved region of the *rimM* gene was selected for primer design to ensure consistent and reproducible amplification of *X. fastidiosa*. The other genes considered had unacceptable homology to non-target organisms in silico or were unable to support LAMP primer design. During the initial primer screening, LAMP primer sets for the disulfide isomerase and *gltA* failed to amplify DNA from many of the *X. fastidiosa* isolates tested and were therefore discarded (data not shown).

Optimization of the rimM LAMP assay. Optimization of the *rimM* LAMP assay indicated that high-performance liquid chromatography (HPLC) purification of the two large inner primers was necessary for reaction efficacy and sensitivity, as previously observed by Varga and James (37). The remaining outer and loop primers functioned with standard desalted purification. Trehalose was examined as an alternative to betaine in the reaction; however, it proved unable to support LAMP amplification and, therefore, was discarded (data not shown). Optimized reaction conditions are given in the Methods section.

Using a serial dilution of *X. fastidiosa* DNA (diluted in *V. vinifera* DNA extract) and varying the incubation time from 45 to 90 min, an incubation time of 60 min at 65°C was found to be sufficient to amplify all isolates tested and consistently amplify a dilution of 500 copies of template per reaction. Inconsistent amplification was sometimes observed with a starting dilution of 250 copies; however, increasing incubation to 75 min improved the reliability of DNA amplification (Table 3). Extending the incubation time past 75 min gave no further increase in sensitivity. Agarose gel electrophoresis of the *rimM* LAMP products produced a ladder pattern with a high-titer band of the major amplification product (180 bp) and several faint, larger products (Fig. 1). This banding pattern is typical when using HPLC-purified primers; use of non-HPLC-purified primers results in a lower titer of the main amplification product and more non-specific laddering (data not shown). Direct sequencing of the major amplification product using the loop primers indicated that the primers were specific to bases 106,752 to 106,862 of the *X. fastidiosa* isolate 9a5C genome (NC_002488), within the *rimM* gene as designed.

Evaluation of hydroxynapthol blue. A color change caused by successful amplification of *X. fastidiosa* in the presence of HNB dye was readily distinguishable, with a clear shift from purple to a light blue (Fig. 2). PCR products from the HNB reaction were also run out on a gel for comparison (data not shown). There was no noticeable inhibition caused by the presence of the dye for samples >500 copies of template per reaction, with a color shift being observed within 60 min. Lower concentrations of template, although producing the typical ladder pattern of successful amplification when examined by gel electrophoresis, did not trigger an identifiable color shift. The results showed that a minimum amount of template (250 to 500 copies) was required to trigger the color change.

Real-time PCR optimization. The real-time PCR assay targeting the *rimM* ORF was found to be robust over a range of annealing temperatures of 58 to 66°C, although the highest reaction efficiency (94.7%, $r^2 = 0.993$) was obtained with an annealing or extension stage of 62°C. The addition of BSA was necessary to reduce inhibition from grapevine leaf samples (data not shown). The detection limit was observed to be ≈10 copies/reaction but there was considerable variation (standard deviation > 1 cycle) between replicate Ct values for samples at this concen-

tration. A concentration of 125 copies per reaction was the minimum titer needed for consistent amplification (standard deviation < 0.5 cycle), with an average Ct value of 31.67 ± 0.39 cycles. The *X. fastidiosa rimM* real-time assay was comparable with the assay of Francis et al. (7) with regard to copy number detection but there was a decrease in Ct of 0.4 to 5.54 cycles for the *rimM* assay for many samples, suggesting that this target region amplifies more readily or that it is less susceptible to inhibitors present in extracts. The *rimM* assay also amplified two samples (Table 2) not detected by the assay of Francis et al. (7).

Comparison to extant methods. The specificity and sensitivity of the *rimM* LAMP and real-time assays compared favorably with the existing conventional (20) and real-time PCR (7) assays. Both the LAMP and *rimM* real-time assay amplified DNA from all 20 *X. fastidiosa* isolates tested. The pear leaf scorch isolate was not amplified by either assay. In comparison, the conventional PCR and Francis et al. (7) real-time assay amplified only 12 and 18 of the 20 *X. fastidiosa* isolates, respectively (Table 2). The pear leaf scorch isolate was amplified by the conventional PCR but not by the Francis et al. (7) real-time PCR. *X. fastidiosa* was successfully detected from infected insect vectors by all assays (Table 2). No amplification was detected from nontarget bacterial species or healthy host species by either the LAMP or real-time PCR assays developed in this study.

For the LAMP and *rimM* real-time assay, amplification of *X. fastidiosa* DNA was successfully achieved from total DNA extracted from a range of infected plant hosts, from infected insect vectors, and from cultured bacterial cells. Although there was no identifiable inhibition from host tissue, the target titer in these samples was well above the limit of detection, with between 4×10^3 and 2.4×10^5 copies/µl as determined by real-time PCR. For the LAMP assay (in the absence of the HNB dye), the limit of detection was ≈250 copies of template per reaction, compared with 10 copies for both real-time PCR assays and 500 copies for conventional PCR (Table 3); the conventional PCR results indicated a sensitivity to inhibitors present in *V. vinifera* extracts in particular, because it successfully amplified similar isolates from culture extracts but not from grape.

TABLE 2. Detection of *Xylella fastidiosa* from infected and healthy host plants, infected insects, and non-target bacterial species using the loop-mediated isothermal amplification (LAMP) and real-time and conventional polymerase chain reaction (PCR)

Bacterial species	Host plant or insect species	Country of origin	Isolate	Source[a]	*rimM*[b] LAMP	*rimM*[b] PCR[c]	*rimM* PCR[d]	Real-time PCR[e]
Xylella fastidiosa								
subsp. *fastidiosa*	*Vitis vinifera*	United States	DSMZ-10026	DSMZ, Germany	+	+	14.50 ± 0.05	15.03 ± 0.07
	V. vinifera	United States	PD0001	L. Nunney, UC Riverside	+	–	24.05 ± 0.05	25.76 ± 0.22
	V. vinifera	United States	PD0004	L. Nunney, UC Riverside	+	+	26.95 ± 0.21	25.76 ± 0.15
	V. vinifera	United States		R. Almeida, UC Berkley	+	–	19.84 ± 0.05	19.25 ± 0.05
	V. vinifera	United States		R. Almeida, UC Berkley	+	–	20.86 ± 0.01	20.46 ± 0.18
	Prunus dulcis	United States	ALS0005	L. Nunney, UC Riverside	+	+	26.20 ± 0.03	28.69 ± 0.05
	P. dulcis	United States	ALS0095	L. Nunney, UC Riverside	+	+	22.32 ± 0.08	25.54 ± 0.57
	P. dulcis	United States	ALS0096	L. Nunney, UC Riverside	+	+	23.26 ± 0.01	25.39 ± 0.00
	V. rotundifolia	United States		C. Chang, UG	+	-	24.54 ± 0.18	26.29 ± 0.67
	Graphocephala atropunctata	United States		R. Almeida, UC Berkley	+	+	30.52 ± 0.23	28.38 ± 0.10
subsp. *multiplex*	*P. salicina*	United States	ICMP-8375	ICMP, Auckland, New Zealand	+	+	17.34 ± 0.14	13.46 ± 0.25
	P. dulcis	United States	ICMP-6575	ICMP, Auckland, New Zealand	+	+	14.92 ± 0.17	14.98 ± 0.19
	P. dulcis	United States	ALS0003	L. Nunney, UC Riverside	+	+	25.51 ± 0.75	27.71 ± 0.06
	Quercus laevis	United States	OAK0023	L. Nunney, UC Riverside	+	–	25.92 ± 0.23	30.18 ± 0.27
	Q. rubra	United States	OAK0024	L. Nunney, UC Riverside	+	–	29.57 ± 0.11	…
	Q. rubra	United States		C. Chang, UG	+	–	17.82 ± 0.29	20.49 ± 0.00
	Liquidambar styraciflua	United States	LIQ0063	L. Nunney, UC Riverside	+	–	29.45 ± 0.06	…
subsp. s*andyi*	*Nerium oleander*	United States	OLS002	L. Nunney, UC Riverside	+	+	19.35 ± 0.04	22.63 ± 0.27
	N. oleander	United States	OLS008	L. Nunney, UC Riverside	+	+	21.45 ± 0.02	25.21 ± 0.63
	N. oleander	United States	OLS009	L. Nunney, UC Riverside	+	+	27.17 ± 0.22	33.01 ± 0.40
subsp. *pauca*	*Citrus* sp.	Brazil	9a5C	H. Coletta Filho, CDC Brazil	+	+	15.86 ± 0.19	16.04 ± 0.07
Unspecified subsp.	*Pyrus* sp.	Taiwan		C. Su, TACTRI, Taiwan	–	+	…	…
Xanthomonas axonopodis pv. *aurantifolii*	…	Brazil	ICMP 14285	ICMP, Auckland, New Zealand	–	–	…	…
X. campestris pv. *citri*	…	United States	ICMP 10012	ICMP, Auckland, New Zealand	–	–	…	…
	…	New Zealand	ICMP 24	ICMP, Auckland, New Zealand	–	–	…	…
X. arboricola pv. *fragariae*	…	Italy	ICMP 17064	ICMP, Auckland, New Zealand	–	–	…	…
Pseudomonas syringae pv. *persicae*	…	New Zealand	ICMP 7090	ICMP, Auckland, New Zealand	–	–	…	…
Pantoea agglomerans	…	New Zealand	…	MAF Collection, New Zealand	–	–	…	…
Agrobacterium tumefaciens	…	New Zealand	…	M. Pearson, Univ. Auckland	–	–	…	…
Spiroplasma citri	…	United States	…	R. Yokomi, USDA-ARS	–	–	…	…
Healthy host species	*V. vinifera*	New Zealand	…	MAF Collection, New Zealand	–	–	…	…
	V. rotundifolia	United States	…	C. Chang, UG	–	–	…	…
	P. persica	New Zealand	…	MAF Collection, New Zealand	–	–	…	…
	Citrus latifolia	New Zealand	…	MAF Collection	–	–	…	…

[a] UC = University of California, UG = University of Georgia, USDA-ARS = United States Department of Agriculture–Agricultural Research Service.
[b] LAMP detection: + = positive and – = negative.
[c] Minsavage et al. (20). Conventional PCR: + = positive and – = negative.
[d] *rimM* real-time PCR. Real-time PCR results with a crossing threshold value of >38 cycles were considered negative.
[e] Francis et al. (7) real-time PCR. Real-time PCR results with a crossing threshold value of >38 cycles were considered negative.

Testing alternative DNA extraction methods for field use. Two alternative DNA extraction methods for plant tissue were compared with the standard DNeasy (Qiagen) column-based method to establish whether extractions could be done in the field. DNA extracted from infected lyophilized tissue of *V. vinifera, V. rotundifolia*, and *Q. rubra* 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 plant cytochrome oxidase (COX) gene (38); results are presented in Table 4. Neither the COX internal control or *X. fastidiosa* were amplified from DNA extracted using the Extract-N-Amp method (Sigma-Aldrich), whereas the PickPen/Invimag (Bio-Nobile/Invitek) protocol worked effectively. Samples amplified an average of 0.40 cycles earlier for COX and 6.45 cycles earlier for *X. fastidiosa* using the DNeasy (Qiagen) compared with the PickPen (Bio-Nobile) method, suggesting that, although field extraction by PickPen is possible, it may not be as effective as the column-based technique for recovering low titers of *X. fastidiosa* DNA.

DISCUSSION

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 (23, 24) seemed to be an appropriate method, because it has been used to amplify and detect plant-pathogenic bacteria, fungi, viruses, and nematodes (8,15,16,25,35,37) as well as human and animal pathogens (6,14). The reaction can potentially be performed in the field because minimal equipment is needed to run the reaction and positive reactions can be identified visually using colorimetric dyes (9).

Assay design made use of the recent advances in *X. fastidiosa* genomic information (4,5,36). The two assays developed, LAMP and real-time PCR, both target the 16S rRNA processing protein gene (*rimM*) that is conserved between all genomic sequences of *X. fastidiosa* but is sufficiently distant from related xanthomonad species. Many other regions targeted by extant PCR-based detection methods are strain or subspecies specific (2–4,12). In contrast to existing PCR assays, both the LAMP and the new real-time assay detected all 20 *X. fastidiosa* isolates tested, representing the four major subspecies. In addition, the assays were able to detect the bacterium from infected insect vectors. The pear leaf scorch isolate was not amplified by either real-time or LAMP assays, which is unsurprising given the genetic divergence between this and other extant *X. fastidiosa* strains (19) but, curiously, was amplified by the conventional PCR.

The conventional PCR (20) is still used routinely for quarantine purposes in several countries. However, the assay does not detect all isolates of *X. fastidiosa* and the sequences that have been published since its development should be incorporated into the design of new primers (5,36).

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 among LAMP assays designed for plant bacterial and viral pathogens (8,16,25,33,37). 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 titer or of poor DNA quality may be missed. Application of an internal control such as COX would reduce the likelihood of false-negative results caused by the latter. It was 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 (S. J. Harper, *unpublished*).

Both the LAMP and real-time PCR assays are rapid, being able to detect *X. fastidiosa* extracted from infected tissue using a simple magnetic-bead based method in ≈1 h, similar to that described previously for real-time PCR (29,32). The assays diverge considerably in equipment requirements. LAMP can be conducted in a water-bath or heat-block; although real-time PCR can be used in the field (34), this method requires an expensive specialized portable thermocycler. The LAMP method may be assessed using a range of endpoint detection methods, including magnesium pyrophosphate accumulation (22), colorimetric hydroxynapthol blue dye (9), fluorescent intercalating dyes such as SYBR Green (Molecular Probes, Inc., Eugene, OR) or PicoGreen (Molecular Probes, Inc.) (32), precipitation with cationic

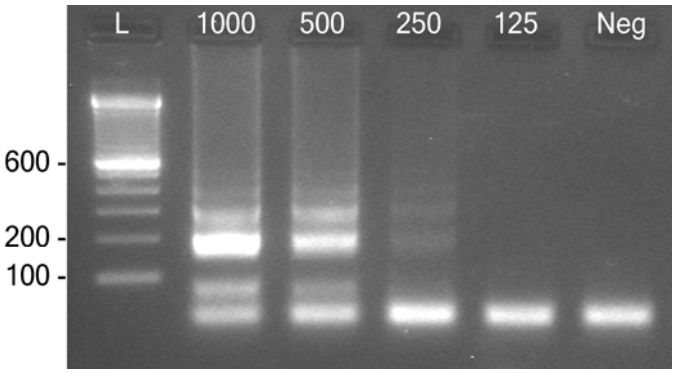

Fig. 1. Results of *rimM* loop-mediated isothermal amplification of serially diluted *Xylella fastidiosa* DNA (copy numbers of 1,000 to 125, diluted in healthy *Vitis vinifera* DNA extract) after 60 min of incubation at 65°C. Neg = healthy *V. vinifera* DNA extract and L = Invitrogen 100-bp ladder.

TABLE 3. Comparison of the sensitivity of the *Xylella fastidiosa rimM* loop-mediated isothermal amplification (LAMP) and real-time polymerase chain reaction (PCR) assays against published real-time and conventional PCR assays

	rimM LAMP[b]								
	45 min		60 min		75 min				
Copies[a]	Gel	HNB	Gel	HNB	Gel	HNB	*rimM* PCR[c]	Real-time PCR[d]	PCR[e]
1,000	+ – –	+ + –	+ + +	+ + +	+ + +	+ + +	28.61 ± 0.09	29.82 ± 0.13	+ +
500	–	–	+ + +	+ + –	+ + +	+ + +	29.64 ± 0.05	30.93 ± 0.07	+ +
250	–	–	+ + –	? – –	+ + +	+ + +	30.52 ± 0.19	31.84 ± 0.11	? –
125	–	–	–	–	+ ? ?	+ – –	31.67 ± 0.39	32.82 ± 0.14	–
10	–	–	–	–	–	–	35.77 ± 1.62	37.84 ± 0.32	–
Negative	–	–	–	–	–	–	–	–	–

[a] Copies per reaction.
[b] LAMP sensitivity: + = positive; – = negative; ? = weakly positive for hydroxynapthol blue (HNB).
[c] *rimM* real-time PCR. Real-time PCR results with a crossing threshold value of >38 cycles were considered negative.
[d] Francis et al. (7) real-time PCR. Real-time PCR results with a crossing threshold value of >38 cycles were considered negative.
[e] Minsavage et al. (20). Conventional PCR: + = positive; – = negative; and ? = weakly positive by gel electrophoresis.

polymers (21), lateral flow devices with labeled primers (35), and gel electrophoresis. Magnesium pyrophosphate detection can be difficult to determine visually (14), while the latter four methods require the tubes to be opened post amplification, yet opening the tubes leads to a risk of aerosol contamination due to the high titer of the LAMP amplicon (31). HNB allows visual detection and is added prior to amplification, which allows the reaction to be performed as a closed-tube system. During this study, HNB required at least 500 copies of target template to trigger color change within an hour; therefore, there is a risk that very low titers of *X. fastidiosa* may not be detected. Increasing the reaction time to 75 min did improve the likelihood of detecting lower concentrations of target DNA; however, reaction times >75 min did not improve the sensitivity of the assay.

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 specialized equipment, and many of the field-based methods proposed are specific to each host–pathogen system (17,34). Being xylem-limited, *X. fastidiosa* presents a particular difficulty for field-based extraction because physical disruption of the tissue or extraction of sap is required (29). In this study, the Extract-N-Amp method which relies on thermal and chemical degradation failed to extract viable DNA, whereas homogenization with a hand-roller and DNA extraction using magnetic beads with a hand-held device (PickPen; Bio-Nobile) was sufficient to extract *X. fastidiosa* DNA from lyophilized samples. Only lyophilized tissue was available for this study; therefore, a comparison could not be made with the sap extraction protocol of Schaad et al. (29). Using the PickPen extraction, the titer 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 for the Qiagen DNeasy extracts, although Ct values for the COX internal control were not markedly different between the extraction types. Such a loss of sensitivity may cause false-negative results for samples of marginal titer. 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 should be investigated.

Finally, the cost of each of the assays tested may be considered. The LAMP assay, with its requirement for specialized enzymes and reagents (especially dNTP usage and the need for HPLC-purified inner primers) costs ≈$5.30 USD (at time of writing) compared with ≈$1.00 USD for conventional and real-time PCR. However, this does not include the cost of specialized equipment such as real-time thermocyclers. The cost of LAMP may be a limitation for large-scale surveys and, for such applications, real-time PCR may be more cost effective. Despite this, LAMP offers a time-saving advantage if reactions are to be carried out in the field, and using LAMP in situ may reduce the need to move infected tissue across country for laboratory testing.

Both the LAMP and the *rimM* real-time PCR assays have 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 using LAMP as a closed-tube assay (31) with colorimetric reporter dyes such as HNB, LAMP has the

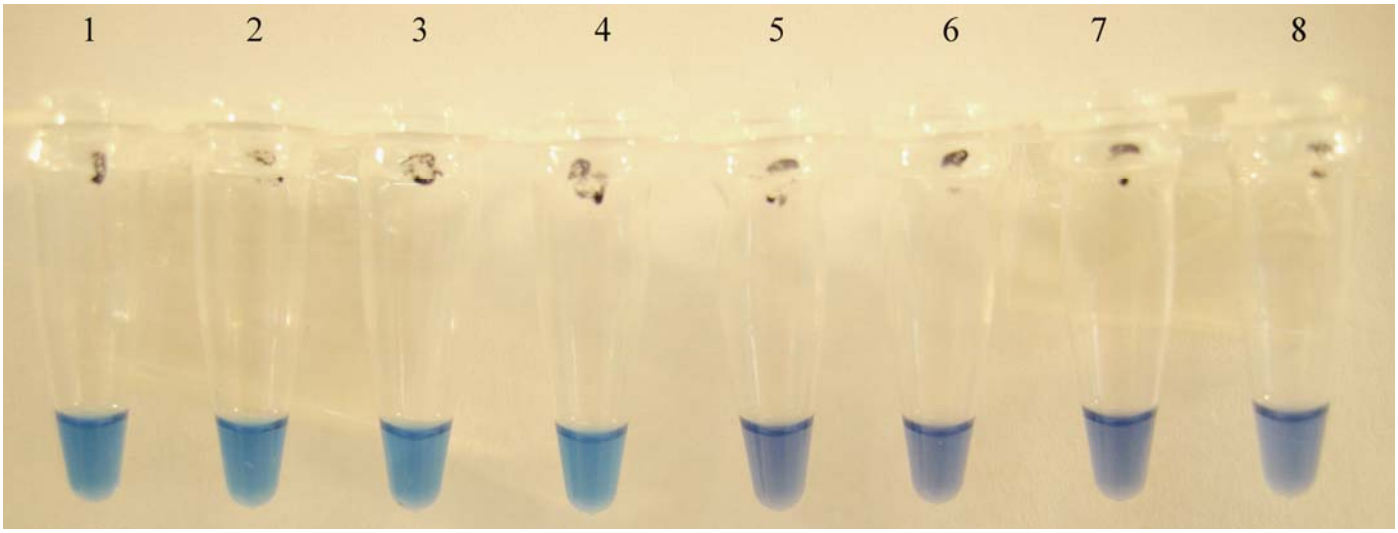

Fig. 2. Successful *rimM* loop-mediated isothermal amplification visualized using hydroxynapthal blue dye showing the sky-blue color change (tubes 1 to 4) observed with *Xylella fastidiosa*-positive samples. Negative samples in which no amplification occurred remain violet (tubes 5 to 8).

TABLE 4. Comparison of field-based extraction methods (BioNoble PickPen or Invimag DNA reagents and Sigma-Aldrich Extract-N-Amp) to the Qiagen DNeasy method, assessed using *Xylella fastidiosa rimM* loop-mediated isothermal amplification (LAMP) (+ = positive and – = negative) and real-time polymerase chain reaction (PCR) assays and a cytochrome oxidase (COX) internal control assay to show PCR competency

		Test		
Source of material	Extraction method	LAMP	Real-time PCR[a]	COX[a]
Vitis vinifera cv. Cabernet Sauvignon	DNeasy	+	20.86 ± 0.01	16.63 ± 0.16
	PickPen w/Invimag Kit	+	28.45 ± 0.11	20.00 ± 0.49
	Sigma Extract n' Amp	–	…	…
V. rotundifolia	DNeasy	+	24.54 ± 0.18	21.23 ± 0.22
	PickPen w/Invimag Kit	+	30.19 ± 0.03	20.83 ± 0.03
	Sigma Extract n' Amp	–	…	…
Quercus rubra	DNeasy	+	17.82 ± 0.29	17.59 ± 0.03
	PickPen w/Invimag Kit	+	22.39 ± 0.22	16.17 ± 0.53
	Sigma Extract n' Amp	–	…	…

[a] Real-time PCR results with a crossing threshold value of >38 cycles were considered negative.

potential to be used in the field. Initial work comparing extraction methods suggests that there is a suitable extraction method that can be used alongside LAMP in situ. The method described here should be readily transferable to other laboratories due to the fact that expensive specialized equipment is not required. It is estimated that a reasonable number of samples (>50) could be processed or screened within 2 h in the field.

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Erratum

The probe sequence was corrected on page 1283, Table 1, to TCGCATCCCGTGGCTCAGTCC. Changes to this article were made on May 16, 2013.