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A rapid field detection system for citrus huanglongbing associated '*Candidatus* Liberibacter asiaticus' from the psyllid vector, *Diaphorina citri* Kuwayama and its implications in disease management

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ABSTRACT

'*Candidatus* Liberibacter asiaticus' (Las), associated with citrus huanglongbing (HLB or citrus greening) is spread by *Diaphorina citri* Kuwayama, the Asian citrus psyllid. Psyllids can be tested to assess the prevalence of Las in a population and for early detection of HLB in new areas being invaded by the psyllid. In some cases, large numbers of psyllids may need to be tested, thus there is a need for rapid and inexpensive field detection methodology. We report here on the development of a field detection kit for testing psyllids for Las using loop-mediated amplification technology (LAMP). Six samples with pools of 1–10 psyllids plus a positive and negative control can be tested at a time in about 30 min; 10 min for crude extraction and 20 min for target DNA amplification. The LAMP assays are conducted in a Smart-DART™ detection unit which is operated from an Android device. The LAMP detection method for Las is about 100 times more sensitive than the traditional real time PCR method. In addition to field testing of psyllids for Las, the methodology was validated as effective for identifying Las in plant DNA extractions. In California, where the psyllid has only recently invaded, participation of a large number of growers and extension workers in field detection may facilitate rapid containment efforts should Las be found. In areas where the disease epidemic is already in the initial stages, large scale testing can be helpful for effective disease management. The LAMP technology lends itself well in such situations.

1. Introduction

Citrus huanglongbing (HLB) or greening is a very severe disease and has caused concern among citrus industries worldwide (Bové, 2006; da Graça, 1991; Halbert and Manjunath, 2004). The disease has been known in the Indian sub-continent for over a century (Crawford, 1912; Husain and Nath, 1927). In the United States, HLB is now established in Florida and has resulted in substantial economic losses, estimated to be about US$3.6 billion in economic activity, in a 5 year period (Hodges and Spreen, 2012). Because of the significant financial implications associated with HLB, the citrus industries and the regulatory agencies in USA, Brazil, and other countries, are interested in early, rapid detection of the pathogen and subsequent management strategies required to mitigate the disease.

Three fastidious gram negative bacteria have been associated with citrus HLB: '*Candidatus* Liberibacter asiaticus' (Las), '*Candidatus* Liberibacter americanus' (Lam) and '*Candidatus* Liberibacter africanus' (Laf). Las is the most prevalent HLB-associated bacterium in Asia as well as in the Western hemisphere. Asian citrus psyllid (ACP; *Diaphorina citri* Kuwayama), the vector of Las has been reported from most citrus growing regions. The first report of ACP in the United States was from Florida in 1998 (Halbert et al., 2000). In Brazil, the psyllid vector prevailed for about 60 years without the pathogen and did not cause significant damage to the citrus industry (Bové, 2006; Lima, 1942).

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Suggested actions for mitigation of citrus HLB include: a) planting of disease-free nursery stock, b) constant scouting for visual detection of symptomatic trees and subsequent removal and, c) control of psyllid vector by pesticide sprays (Belasque et al., 2010; Bové, 2006; Grafton-Cardwell et al., 2013; Hall et al., 2013). Starting a citrus grove with HLB-tested disease-free nursery stock is an excellent method of disease control and is currently being implemented by regulatory agencies in the United States and Brazil. Reduction of inoculum by removing infected plants based on visual detection of HLB symptoms was followed in many citrus industries including Brazil (Belasque et al., 2010; Bové, 2006). It has been shown that infected plants can remain non-symptomatic for an extended period of time, and hence tree removal will not be very effective since the pathogen is known to have a lengthy incubation and latent period (Chiyaka et al., 2012; Gottwald, 2010). In several locations in Florida, Las was first recorded in psyllids and the subsequent detection in field plants was verified 6 months to 3 years after the initial find in psyllids (Manjunath et al., 2008). Under controlled conditions, Pelz-Stelinski et al. (2010) have demonstrated that it may take one year or longer to detect Las in plants that are successfully inoculated by Las-positive *D. citri*. HLB disease management based on constant monitoring of the psyllids for Las may be a suitable approach.

A large number of psyllids could be tested for the presence of HLB associated bacteria if simple testing kits become available. Currently accepted methods of detection include quantitative real-time PCR (qPCR) testing for the 16S rDNA region of Las followed by conventional PCR to amplify a larger region of this gene (Jagoueix et al., 1996). Sequencing of the amplicon and significant identity with known Liberibacter sequences are deemed confirmatory. From a disease management perspective, rapid detection of the pathogen either in the plant or in the psyllid vector is useful for implementing pathogen exclusion strategies for intra-orchard disease mitigation. Instant detection of the pathogen would facilitate implementation of required management practices in a timely fashion. While a positive adult psyllid would indicate the presence of the pathogen in the area, a positive nymph would mean that the source tree is infected. Field detection capabilities would enable the extension workers or grove managers to alert the regulatory agencies to execute prevention and/or suppression operations in certain regions of high priority. It is important to note that any significant result using a field detection system needs to be confirmed by further testing in a regulatory or research laboratory for final confirmation.

Loop-mediated isothermal amplification (LAMP) is a very simple, cost-effective and sensitive technique for detection of specific DNA sequences, first described by Notomi et al. (2000). In LAMP, isothermal amplification is conducted with 4–6 primers (Supplementary Fig. 1). Since six primers specific to eight distinct regions are used for LAMP, amplicons generated are very specific (Notomi et al., 2000; Tomita et al., 2008). LAMP does not require expensive thermo cyclers, sophisticated laboratory facilities or trained scientific personnel. The enzyme utilized, *Bst* DNA polymerase (or similar enzyme), is capable of autocycling strand displacement DNA synthesis. LAMP has been shown to be highly resistant to interferences from biological contaminants (Kaneko et al., 2007) and, hence, simple and inexpensive template preparation methods are often sufficient for enabling detection of target DNA. LAMP has been successfully utilized to detect a wide variety of targets, such as potato spindle tuber viroid (Lenarcic et al., 2013), bacterial wilt caused by *Ralstonia solanacearum* (Kubota et al., 2008), citrus canker caused by *Xanthomonas citri* subsp. *citri* (Rigano et al., 2010), zebra chip disease of potato associated with '*Candidatus* Liberibacter solanacearum' (LSO; Levy et al., 2013; Ravindran et al., 2012), and Pierce's disease caused by *Xylella fastidiosa* (Harper et al., 2010).

The LAMP assay previously described for detecting HLB-associated Las from citrus tissue using a *tuf*B-*sec*E-*nus*G-*rpl*KAJL-*rpo*B gene cluster (Okuda et al., 2005) was found to be about 100 times less sensitive than qPCR method developed for 16S rDNA region (Li et al., 2008). Rigano et al. (2014) have developed an LAMP method combined with a lateral flow dipstick assay to detect a hypothetical protein region from Las. Ravindran et al. (2012) have described an LAMP method to detect Las using primers for the 16S rDNA of the pathogen. However, for detection of HLB associated Las, LAMP has not been used widely so far. The availability of the full genome sequence of Las (Duan et al., 2009) has enabled researchers to evaluate other regions of the bacterium that are more suitable for PCR-based detection technologies (Morgan et al., 2012).

We have developed a rapid, cost-effective, easy to operate, and field deployable technique to detect Las in psyllids. The method is very simple and can be routinely used effectively by citrus growers, extension workers and home owners. It would be very useful to have quick and simple diagnostic tools to detect the pathogen in psyllid vectors in citrus growing regions of the world where facilities are not available for expensive PCR testing. In addition, growers could afford regular monitoring of their groves. Extension workers and inspectors will have information that would enable them to alert a local testing laboratory if positive psyllids are detected. The first report of HLB in Louisiana was triggered by a report from a home owner who spotted a psyllid on a "symptomatic tree" (Hummel and Ferrin, 2010). Utilizing the methodology and the instrumentation described in this work, we envision potential for implementing a wide surveillance program for detection of the pathogen. Rapid and reliable testing of a large number of psyllids combined with traditional methods of control, including targeted pesticide sprays to eliminate Las-positive psyllid sub-populations, will enable efficient and financially sustainable HLB management strategies.

2. Materials and methods

2.1. Source of psyllids

Psyllids maintained on HLB infected plants in an insectary at the USDA ARS, United States Horticultural Research Laboratory, Fort Pierce were used for development of LAMP technology. Preserved psyllids stored in 95% ethanol were obtained from psyllid-infested regions of Florida, Brazil and Pakistan for testing in California. Las-free *D. citri* were obtained from a psyllid colony maintained at the quarantine facility located in the Dept. of Entomology, University of California Riverside (UCR), CA. Samples of the tomato psyllid, *Bactericera cockerelli* maintained on tomato plants were obtained from Dept. of Entomology, UCR, CA.

2.2. Extraction of psyllid DNA

For the LAMP assay, crude ACP extracts were prepared as follows; 1–20 psyllids were removed from the collection tubes, the ethanol was air-dried on a piece of filter paper for 2–5 min and the psyllids were dropped into individually capped PCR tubes containing 100 μL of extraction buffer (20 mM Tris, pH 8.0 containing 2 mM EDTA and 1% TritonX100®) and heated in the Smart-DART™ unit for 10 min at 85 °C. The samples were centrifuged for 5 s in a micro-centrifuge and the clear supernatant was used for the LAMP assay. The tomato psyllids (*B. cockerelli*) carrying '*Candidatus* Liberibacter psyllaurous' (synonym, *Ca.* L. solanacearum), a heterologous bacterium associated with tomato psyllid yellows (Hansen et al., 2008), were used as negative controls in LAMP assays. For a comparative qPCR testing of Las from the psyllids, extractions were conducted using a Qiagen® Magmax kit (Qiagen Inc. CA). The qPCR

reactions were conducted with primers and TaqMan™ probes for the psyllid internal control gene 'wingless' and the 16S rDNA fragment from Las (Manjunath et al., 2008).

2.3. Extraction of plant DNA

Plant samples were obtained from field trees of many cultivars of citrus and close relatives from a severely HLB affected area in Florida. Plant DNA extracted using Plant DNeasy kit from Qiagen® was used for LAMP assay, mainly to validate the LAMP protocol and to compare the results with qPCR assays conducted from the same extractions.

2.4. PCR probe and primer design to detect phage-related gene fragment by LAMP

We have selected a 177 bp DNA fragment of Las encompassing a phage related genomic region (Tomimura et al., 2009). The target region consisted of 111 bp from the 3′ terminus of CLIBASIA_00025 (annotated as ABC-type dipeptide transport system, periplasmic component), 3 nucleotides from the intergenic region and 63 bp from the 5′ terminus of an adjacent gene, CLIBASIA_00030 (putative DNA polymerase of bacteriophage origin). This 177 bp sequence is conserved in many isolates of Las described from Southeast Asia (Tomimura et al., 2009). All the publicly available Las sequences for the 177 bp target region were aligned and confirmed to be highly conserved in Las strains from different geographical regions. The primers F3, B3, F1P and B1P required for LAMP were designed using Primer explorer version 3 software (http://primerexplorer.jp/e/). The loop primers LF and LB were designed manually (Table 1, Supplementary Fig. 1). Primers were synthesized by Integrated DNA technologies, Coralville, IA, USA and the two double-domain primers, F1P and B1P, were HPLC purified. The specificity of the primers was checked *in silico* against all available sequences in the Genbank.

2.5. Smart-DART™ and Android device

We have used the Smart-DART™ tool from Diagenetix Inc.™ for our experiments. The platform includes a custom device that can analyze 8 samples simultaneously, running at a programmable temperature, and periodically measuring fluorescence. The Smart-DART™ device interfaces wirelessly (by Bluetooth®) to an Android device through a custom application, which allows the user to control the reaction settings and view data graphically in real time (Fig. 1). Fluorescence readings were recorded using the channel optimized for fluorescein. Reactions were conducted in strips of 8 optically clear tubes that can be individually capped with a seal and lock mechanism to avoid cross contamination. The Smart-DART™ platform was used for psyllid DNA extraction (at 85 °C for 10 min) as well as for the LAMP reaction for detection (at 65 °C for 20 min). The results can be saved to view later, or e-mailed from the Android device. The platform functions as a closed amplification and detection system which limits the risk of amplicon contamination of the work area. For comparison to benchtop laboratory instruments, the reactions conducted using the Smart-DART™ unit were also repeated using a Stratagene qPCR machine with the same nominal conditions.

2.6. LAMP methodology

For detection of the target region for Las selected for LAMP assay in both Smart-DART™ and Stratagene qPCR machine, we followed the conditions outlined in Table 2. We used ISO-001 master mix (obtained from Optigene® Limited, West Sussex, United Kingdom) for LAMP assays. The master mix contains an engineered GspSSD LF DNA polymerase (*Geobacillus* sp. SSD, large fragment DNA polymerase) with strand displacement and reverse transcriptase activities and without any exonuclease activity. Detection of fluorescence of the amplification product was achieved using the Smart-DART™ device. The enzyme mix used for the assays also has a recombinant pyrophosphatase from *Aeropyrum pernix* and hence the reaction does not produce adequate quantities of inorganic phosphate in the buffer. Turbidimetric detection of the amplicons is not an option under these conditions. LAMP reactions were conducted in 20 μL volume in 250 μL PCR tubes with individual caps. The reaction mix consisted of the six primers, Optigene® master mix and insect or plant DNA template, and amplification was conducted in the Smart-DART™ unit for 20 min at 65 °C (Table 2).

For validating LAMP assays, simultaneous LAMP and qPCR assay of DNA extractions of psyllid and plant samples were conducted (Li et al., 2006; Manjunath et al., 2008). Crude extracts used in LAMP assays were not suitable for qPCR assays, and hence an additional purification step using Qiagen columns was included.

2.7. Analysis of LAMP products

LAMP products were analyzed by two methods: a) electrophoresis and b) fluorescence measurement using Smart-DART™ software. Electrophoresis was conducted using 4 μL of the amplification product from the LAMP reaction at 100 V for 45 min on 2% agarose gels. The gels were stained with ethidium bromide and photographed. Synthesis of new DNA by LAMP results in incorporation of the proprietary DNA binding dye included in the Optigene® master mix. The dye has an excitation maxima at 490 nM and an emission maxima at 525 nM; the Smart-DART™ device records fluorescence in real time. Samples were classified as positive if there was a sustained increase in observed fluorescence exceeding a threshold value relative to the initial background noise. Samples classified as positive are assigned a threshold time equivalent to the time at which the peak rate of increase in fluorescence occurs (time of positivity or, t_p).

Table 1
Primers used for LAMP assay for detection of '*Candidatus* Liberibacter asiaticus'.

Primer	Sequence	Location[a]	Orientation	Length	Tm	GC(%)
F3	GAAATCGGACACTTCGGAGTT	3630-3650	F	21	56	47.6
FIP(F1c-F2)[b]	ACCTCCGCTGAGGCAAAGTTTG - TAAGGATTACGGCGAAGAGCA	3713-3692; 3651-3671	R-F	43	73.6	51.2
LF	AGACCGAGGTATCGGAGTCT	3691-3672	R	20	57.1	55
B3	ACCCCTACCTTAGGCAAGG	3806-3788	R	19	56.2	57.9
BIP(B1c-B2)[c]	GCTCTGCCGTGGAACTGATCA - GTTGAGGACTGCGGGTTTC	3724-3744; 3787-3769	F-R	40	73.4	57.5
LB	ATCAATGTCAAAGCTGTTTATCGACATT	3741-3768	F	28	58.9	32.1

[a] Location of the primer with reference to complete genome of '*Candidatus* Liberibacter asiticus' (Genbank sequence CP_001677.5).
[b] Forward internal primer, FIP consists of two fragments, F1c and F2, separated by a hyphen in the sequence column. They are in reverse (F1c) and forward (F2) orientations.
[c] Backward internal primer, BIP consists of two fragments, B1c and B2, separated by a hyphen in the sequence column. They are in forward (B1c) and reverse (B2) orientations.

Fig. 1. Smart-DART™ technology for detection of *Candidatus* Liberibacter in citrus psyllids. A custom app installed on an Android device (A) is used via Bluetooth® to operate the Smart-DART™ (B). The lid of the unit is partially opened to show the 8 reaction tubes inside. Psyllids are air-dried at 25 °C for 2–5 min (C) and dropped into extraction tubes (D) and incubated in Smart-DART™ at 85 °C for 10 min to extract crude DNA. 5 μL of the extract is transferred to reaction tubes (E) and the LAMP reaction is carried out in Smart-DART™ at 65 °C for 20 min. Amplification graphs are visualized on the Android device which records the data as comma delimited values which can be shared by e-mail or viewed later within the app.

2.8. Standard curve for LAMP assay using a cloned target

For evaluation of the sensitivity of LAMP technology, we have constructed a synthetic clone containing a 456 bp fragment of Las (corresponding to nucleotides 1218932-1218476 of Las genome sequence of the psy62 strain from Florida, Genbank accession no. NC_012985) in pUC57 vector. The DNA concentration of the plasmid construct was measured three times using a Nanodrop 1000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). The LAMP experiments were conducted in triplicate and the average t_p values were plotted. To assess the efficiency of amplification, an alternative analysis to PCR amplification efficiency (Li et al., 2008) was used. Because LAMP amplification is a continuous process that results in a growing concatenation of amplicons rather than discrete individual copies over well-defined control cycles as in qPCR, we instead estimated a "doubling time" τ for the LAMP process based on observed t_p. This analysis assumed that reactions resulted in exponential rates of DNA polymerization, and that t_p corresponded to the time when a constant repeatable threshold quantity (K) of double stranded DNA was produced. Mathematically,

$$K = c_i 2^{t_p/\tau} \quad (1)$$

Table 2
Reaction conditions for LAMP assay using Smart-DART™ and Stratagene qPCR.

Reagent	Final concentration
OptiGene master mix ISO-001	1X
Primer FIP	1.6 μM
Primer BIP	1.6 μM
Primer F3	0.2 μM
Primer B3	0.2 μM
Primer LF	1.6 μM
Primer LB	1.6 μM
Template DNA	5 μl
Total reaction volume	50 μl
Reaction conditions for LAMP in Smart-DART	Process
85 °C for 10 min	Extraction of psyllid DNA
65 °C for 20 min	LAMP cycling, detection
Reaction conditions for LAMP in qPCR machine	Process
65 °C for 20 min; 20 cycles	Amplification, detection

where c_i is the initial template DNA quantity. Through a simple manipulation of Equation (1), the doubling time can be inferred from the relationship between t_p and c_i:

$$t_p = \frac{\tau}{\log(2)}[\log(K) - \log(c_i)] \quad (2)$$

The doubling time τ then can be estimated as the product of $-\log(2)$ and the slope of t_p vs $\log(c_i)$.

3. Results

3.1. Amplification of the Las target region using LAMP

An amplicon of 137 bp and multimers of this product were synthesized in the LAMP reaction (Supplementary Fig. 1). The LAMP reactions were scored based on the time of positivity (t_p). Most positive samples showed an amplification plot in less than 10 min in the LAMP assay. We use a t_p value of 15 as the cut-off to determine positive or negative samples.

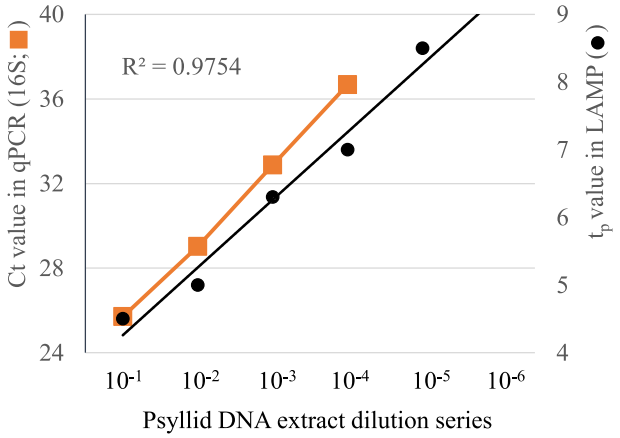

Fig. 2. Comparison of qPCR and LAMP testing. Psyllid DNA extract known to be positive for Las was serially diluted to obtain 10^{-1} to 10^{-6} dilutions. Ct values for qPCR of 16S rDNA region and t_p values for LAMP assay of phage related region are plotted. qPCR worked in 4 dilutions (3 considered positive) and LAMP worked in 5 dilutions (all considered positive). R^2 value of standard curve obtained from LAMP assay is indicated.

3.2. LAMP is about 100 times more sensitive than qPCR for detection of Las from psyllids

We compared the standard qPCR assay (conducted routinely in many labs for the 16S rDNA region) with the LAMP method (for the phage region) using aliquots of the same extractions from a batch of known Las-positive *D. citri*. Ten-fold serial dilutions (10^{-1} to 10^{-6}) of the plasmid DNA were utilized for qPCR and LAMP analysis. The qPCR cycle threshold values (Ct) of the first three dilutions were below 33 and these samples were considered as positive for Las; dilution no. 4 had a Ct of 36 which is generally regarded as negative. In the LAMP assay, the first 5 dilutions had a t_p value of 4–8.5 indicating that they were positive (Fig. 2).

3.3. LAMP can detect one positive psyllid from a pool of 10 psyllids

We pooled Las-positive psyllids with Las-negative psyllids to test if the LAMP method can detect single positive insects from a pool of negative psyllids. Las positive psyllids obtained from the Las-positive *D. citri* colony (from Fort Pierce, FL) were first evaluated by testing single psyllids by qPCR for 16S rDNA fragment to estimate the percentage of positives. About 29% of the psyllids from this particular batch were positive for Las (data not shown). Single insects from the Las-positive batch of psyllids (from Fort Pierce) were pooled with 0, 4, 9 and 19 psyllids obtained from the UCR quarantine facility (Las-free psyllids). The t_p values of the samples in LAMP assay vary depending on the Las titer in the infected psyllid. When using the binomial distribution (Snedecor and Cochran, 1989) to account for the portion of psyllids from the "infected" colony that were not positive for Las, we found that within a 95% confidence interval the LAMP method could routinely detect a single Las positive psyllid in a pool of up to 20 psyllids (Table 3).

3.4. LAMP assay is quantitative

Typically LAMP positive reactions are detected by visualizing the turbidimetric endpoint (Tomita et al., 2008). LAMP has been demonstrated to be quantitative since a linear increase in turbidity can be correlated with increasing amounts of the initial template (Han et al., 2011; Mori et al., 2001). The ISO-001 reaction mix we utilized has a pyrophosphatase included in the mastermix and hence inorganic phosphate does not accumulate and the reaction does not become turbid. However, since we are using a fluorescence-based platform, we can measure increase in fluorescence over time. We used a DNA sample consisting of the synthetic clone of the LAMP target region, used serial dilutions of the plasmid preparation and recorded the t_p values in LAMP assay. The t_p value increased as the concentration of the plasmid decreased. In the concentration range that we checked, six dilutions of the plasmid sample showed a linear relationship when plotted against t_p. Fig. 3 shows a typical LAMP amplification graph recorded in the Android device connected to the Smart-DART™ unit. The t_p values ranged from 5 to 10.5 for plasmid DNA concentrations corresponding to 2130 to 0.0213 pg of DNA per mL (Fig. 3 A and B). These results suggest an LAMP doubling time of about 0.34 min (~20 s) when testing cloned DNA, a value very similar to that observed for amplification in dilutions of psyllid extract. Similar linearity was observed when psyllid extractions were serially diluted and t_p estimated (Fig. 2).

Table 3
Pooling of Las-positive and negative psyllids and analysis by LAMP.

Ratio no.	Las+ ACP[a]	Las− ACP[b]	Total replicates	Positive replicates	% positive	*p*-value[c]	t_p value[d]
1	1	0	24	9	37.5	0.872	8–14
2	1	4	16	2	12.5	0.115	9–11
3	1	9	16	4	25	0.485	9–12
4	1	19	16	3	18.75	0.274	12–18
5	0	1	16	0	0	*	*

*Not applicable because no infected psyllids were sampled and no amplification was observed.

[a] Psyllids obtained from a Las-positive colony from Fort Pierce, FL, with observed infection rate of 29%.

[b] Psyllids obtained from a Las-negative colony from Entomology Dept., UCR quarantine facility.

[c] Cumulative probability that members selected from a population with the given infection rate will result in the observed number or fewer positive replicates, according to the binomial distribution (Snedecor and Cochran, 1989).

[d] The observed range of values for time of positivity (t_p) for all replicates for the given condition.

3.5. LAMP assay validated with plant tissue from many citrus varieties

Since it is possible that certain plant samples can have inhibitors that affect LAMP reaction, we tested cultivars belonging to 23 accessions (*Citrus* species as well as some closely related genera) by LAMP assay. The plant extractions were made by Qiagen kit and tested by qPCR (for the housekeeping gene, 'Cox' and for 16S rDNA of Las) and by LAMP (phage related region targeted in this study). All the samples that were found to be positive by qPCR were also positive by LAMP. Some plant samples ('South Coast Field Station' citron, Lamas lemon, and Tavares limequat) had higher Ct values (between 31 and 33) for Las in qPCR assay but were clear positives by LAMP assay (t_p value of 8–8.5; Supplementary Table 1). All the clear negative samples with a Ct value of 40 in qPCR were also negative by LAMP.

3.6. Gel electrophoresis of LAMP products

A LAMP reaction results in products with stem-loop structures and several inverted repeats of the target DNA. Cauliflower-like structures with multiple loops are reported (Parida et al., 2008; Kubota et al., 2008). To test if the products made in our LAMP reaction conformed to the expected banding pattern, we electrophoresed the amplification product on a 2% agarose gel. A typical ladder pattern was observed on agarose gels (Fig. 4; the bands with higher molecular weights are multimers of the starting structure for LAMP cycling shown in Supplementary Fig. 1B). Las negative ACP samples and non-target tomato psyllid samples known to be positive for a related heterologous bacterium, LSO did not show the amplification products on gels. LAMP products from the various test runs conducted during this study (pooling healthy and infected psyllids to determine the sensitivity, measurement of linearity using a plamid preparation, testing of heterologous psyllid populations along with Las-positive ACP, testing of HLB-positive plant samples) were scored by t_p values and also evaluated by gel electrophoresis. After the initial validation in the laboratory during development of methodologies, it will not be necessary to conduct electrophoresis of LAMP products on a routine basis. A closed tube assay will reduce chances of contamination.

4. Discussion and conclusions

Early detection capabilities are very important for any disease containment or management. In dealing with human diseases, the World Health Organization has suggested some guidelines for an ideal diagnostic test that can be utilized in situations where financial considerations impede implementation of the required precautionary measures for disease control. To be suitable for

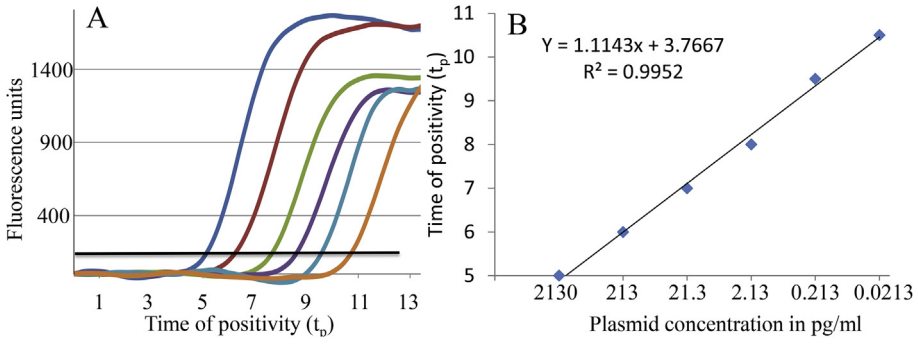

Fig. 3. Standard curve generated in the LAMP assay using plasmid DNA. Panel A. Serial dilutions of plasmid DNA were used for LAMP and t_p values plotted against fluorescence. Panel B. Amount of plasmid (represented as picograms/mL) plotted against t_p values. The slope of the curve and R^2 were calculated using Excel.

resource-limited situations, the tests should be *a*ffordable, *s*ensitive, *s*pecific, *u*ser-friendly, *r*obust, *e*quipment-free and *d*eliverable to the end user (abbreviated as 'ASSURED'; Mabey et al., 2004). For the citrus industry, testing of psyllids for the presence of the pathogen associated with the devastating disease HLB is vital. We believe the technology described here represents a first step towards an 'ASSURED' test deployable in the field for early detection of Liberibacters. We were able to obtain reliable results even when using crude extracts making this method very attractive to growers for use outside a diagnostic lab.

Detection of Liberibacters in psyllids results in an early warning system indicating the impending disease in the plants after a certain period of time (Chiyaka et al., 2012; Manjunath et al., 2008). While psyllid nymphs feeding on asymptomatic, infected trees can be found to be positive for Las, it takes much longer to detect the Liberibacters from infected plants. Psyllid testing can detect the presence of Liberibacters long before infected plants can be found by qPCR assays; however, field validated early detection methods for HLB-positive plants are still not available. Easy to operate field detection kits would enable regulatory agencies to utilize valuable resources in areas requiring immediate attention.

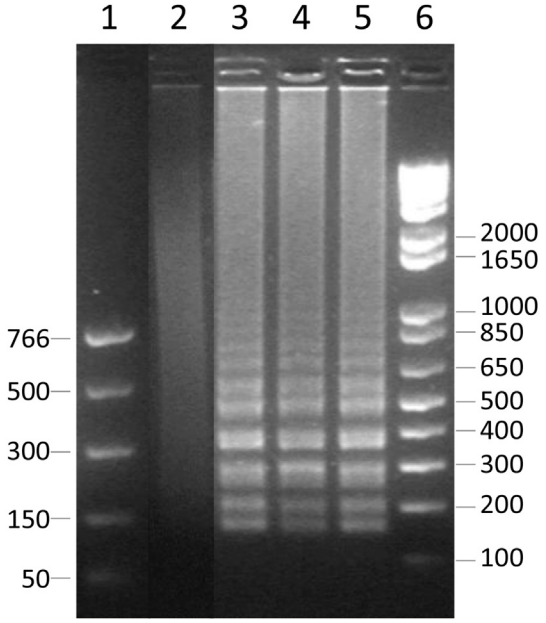

Fig. 4. Agarose gel electrophoresis of LAMP products. Lane 1: PCR molecular weight marker; 2: healthy Dweet tangor (from USDA Repository, Riverside, CA) 3–5: citrus extractions from Florida, Fx20069, 20070, 20071 and lane 6:1 Kb + DNA marker. Electrophoresed on 2% agarose gels, stained with ethidium bromide and visualized.

Psyllid testing is presently used widely for prevention and suppression of HLB in several countries. Testing of psyllids by a limited number of regulatory laboratories may not be able to meet the needs of the citrus community battling the establishment of HLB in many citrus growing regions. Typically, it would take a few weeks to several months for the citrus grower to obtain psyllid testing results from laboratories. Availability of easy to operate, cost effective testing kits that can be widely deployed and used by all interested personnel would facilitate targeted control of Las-positive psyllid populations. Access to instant test results would enable the growers to implement timely orchard management practices.

The commonly utilized bacterial gene used for laboratory based diagnostics of Las is a fragment of the 16S rDNA gene (Li et al., 2006). We have developed LAMP primer sequences for the phage related region of the Las genome. If Las positives are found, the crude extracts used in LAMP assays can be re-evaluated in diagnostic laboratories by qPCR for the 16S rDNA region. Utilization of two different genomic regions will be beneficial in detecting potential contaminations. The LAMP assay developed here is about 100 times more sensitive than standard qPCR technique and hence likely to detect the bacterium in low-titer situations (Fig. 3). While testing a large number of plant DNA samples of different varieties, we observed that it was easier to discriminate between weak positives and negatives in LAMP assays rather than in qPCR assays where samples with Ct values of 34 or above are generally considered inconclusive. Very high levels of sensitivity of LAMP reactions have been reported in many other systems. In the filarial parasite *Loa loa* associated with a tropical human disease Loiasis, it has been shown that LAMP assay can detect 0.5 ag of the worm genomic DNA; compared to the qPCR test that detects 0.1 pg, LAMP was considered 200,000 times more sensitive (Fernandez-Soto et al., 2014). In our analysis, the increased sensitivity observed in LAMP compared to qPCR may be because of two reasons: a) qPCR is generally conducted in a duplex format to detect plant gene ('Cox') or psyllid gene ('wingless') in addition to the bacterial gene (16S rDNA). When the bacterial titer is low, amplification of the internal control genes may deplete the reagents required for the amplification of the bacterial target gene and this may negatively affect the Ct value calculated in qPCR assays; b) when target concentration is low, the PCR inhibitors present in the extract may negatively affect qPCR Ct values. Such an effect seems to be less of a problem with LAMP reactions. When the qPCR assays were conducted for only Las (without housekeeping gene) and the bacterial titers were low, the sensitivity of qPCR appeared to be lower than LAMP.

The Liberibacter genomic region chosen by our study seems to be specific to Las and is not reported from other bacteria. Psyllids are known to harbor several endosymbiotic bacteria like *Wolbachia* (alpha proteobacterial group) and *Candidatus* Carsonella (gamma proteobacteria) (Saha et al., 2012). The LAMP reaction was always negative with known Las-free ACP and *B. cockerelli* indicating that

the primers did not amplify DNA from the endosymbiont populations. If the LAMP technology is extended in the future to test plant root samples that are reported to have the bacterium earlier than the canopy (Johnson et al., 2013), it is imperative that the target region for testing is unique to Las and does not amplify DNA from root-inhabiting Rhizobia. *In-silico* analysis of the target phage region determined that the region selected in this study is specific to Las and not present in other members of Rhizobiales.

We have validated the LAMP protocols described here using psyllids from Florida, Brazil, India and Pakistan (data not shown), as well as from a range of different citrus varieties (Supplementary Table 1). Since LAMP technique is highly sensitive, contamination of samples can potentially become a problem. This can be resolved by a few simple guidelines: a) discarding LAMP reaction tubes after the assay without opening; b) using 8-strip tubes with individual caps, opening and closing one tube at a time; c) cleaning work area, pipets and other plastic ware with 0.1 × commercial bleach; d) using gloves during testing and e) testing psyllid samples in the farm/grove where they are collected rather than transporting all psyllid samples to a central location.

Growers should be encouraged to test pools of psyllids with up to 10 psyllids per extraction. While a positive result would mean the presence of Liberibacter, a negative result may not mean absence of the pathogen. The percentage of psyllids carrying the pathogen is usually low under field conditions and varies greatly in different seasons. Hence, testing of large number of psyllids in different seasons is desirable.

The LAMP technology will be very useful for citrus growing regions where HLB has either not been found, or is in the initial stages of the epidemic. The disease situation in Florida reached alarming proportions in a very short period of time (Gottwald, 2010; Halbert et al., 2012). Pro-active measures in citrus growing regions of the United States where the imminent danger of HLB exists but the disease has not established yet, like California, Texas and Arizona, may assist both HLB prevention/suppression and psyllid management regimen. We believe that large scale testing of the psyllid by many interested parties working together with the regulatory agencies will achieve such a goal. While finding a positive psyllid may not lead to any regulatory actions, the grower can start immediate action to prevent further spread of the pathogen by suppressing psyllid population. Control of psyllids and targeting Las-positive psyllids is a short-term solution to delaying the epidemic and mitigation of the disease. Long-term solution for this devastating disease consists of developing HLB tolerant/resistant cultivars. Till that goal is achieved, other strategic methods of disease control will be useful in disease management.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cropro.2014.10.026

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