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Development of rapid, sensitive and non-radioactive tissue-blot diagnostic method for the detection of citrus greening

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ABSTRACT

Citrus Huanglongbing (HLB or citrus greening) is one of the most devastating diseases of citrus worldwide. The disease is caused by Gram-negative, phloem-limited α-proteobacterium, '*Candidatus Liberibacter asiaticus*', vectored by the psyllid, *Diaphorina citri* Kuwayama. Citrus plants infected by the HLB bacterium may not show visible symptoms sometimes for years following infection and non-uniform distribution within the tree makes the detection of the pathogen very difficult. Efficient management of HLB disease requires rapid and sensitive detection early in the infection followed by eradication of the source of pathogen and the vector. The polymerase chain reaction (PCR) based method is most commonly employed for screening the infected/suspected HLB plants and psyllids. This is time consuming, cumbersome and not practical for screening large number of samples in the field. To overcome this, we developed a simple, sensitive, non-radioactive, tissue-blot diagnostic method for early detection and screening of HLB disease. Digoxigenin labeled molecular probes specific to '*Ca. L. asiaticus*' nucleotide sequences have been developed and used for the detection of the pathogen of the HLB disease. The copy number of the target genes was also assessed using real-time PCR experiments and the optimized real-time PCR protocol allowed positive '*Ca. L. asiaticus*' detection in citrus samples infected with '*Ca. L. asiaticus*' bacterium.

1. Introduction

Citrus Huanglongbing (HLB or Citrus greening) is one of the most severe diseases of citrus occurring in citrus growing Asian countries, Africa, Brazil and USA. The HLB disease is caused by a Gram-negative, non-cultured, phloem-limited α-proteobacterium which is transmitted by psyllid vectors [1,2]. Three species of HLB, viz., '*Candidatus Liberibacter asiaticus*', '*Candidatus Liberibacter africanus*' and '*Candidatus Liberibacter americanus*', have been identified which differ in their vector specificity and environmental conditions. '*Ca. L. asiaticus*', a heat-tolerant species, and '*Ca. L. africanus*', a heat-sensitive species, are vectored by both *Diaphorina citri* Kuwayama and *Trioza erytreae* Del Guercio, while '*Ca. L. americanus*', a heat-tolerant species, is vectored only by *D. citri* Kuwayama [1,3,4]. HLB causes a rapid tree decline characterized by yellowed shoots with blotchy mottled leaves or variegated type of chlorosis giving the impression of nutrient deficiencies, small, poor quality lopsided fruit with color inversion, aborted seed, and over time it debilitates the productive capacity of citrus trees [1,5,6].

Anatomical studies of HLB-affected sweet orange shoots with '*Ca. L. africanus*' indicated the association of disorder of the phloem and massive accumulation of starch in the plastids [7]. However, it is unknown how *Ca. Liberibacter* spp. spread throughout the tree and cause the disease. In the infected trees the bacterium is distributed unevenly in bark tissue, leaf midribs, roots and floral parts, often in an erratic manner even within the same tree. Sometimes some branches show the symptoms while the adjoining branches almost look healthy with low concentration of the bacterium below the detection threshold. Hence, there is a greater

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need to develop methods for the detection of the pathogen to formulate management strategies to combat the citrus greening. It is very important to detect the HLB bacterium from early infected citrus trees prior to visible greening symptoms. Due to low concentrations and uneven distribution of HLB in the infected tree, the detection of the HLB bacterium in symptomless tree is a formidable task [8–10]. DNA probes, enzyme-linked immunosorbent assay, electron microscopy, and biological assay have been used [5,11–13] for the detection of HLB. However, polymerase chain reaction (PCR) based detection method is the most commonly employed method of screening and is used routinely to confirm the HLB infection from suspected samples [10,14]. Although this method is very efficient and specific, false negatives and false positives are common due to low titer of bacteria inside the phloem, presence of PCR amplification inhibitors in the phloem sap and non-specific DNA amplification by PCR [10]. It is also not practical to rapidly screen thousands of samples in the field. Therefore, a simple and sensitive broad-spectrum assay would be more useful for the detection of HLB from large number of field samples. We developed a rapid and large-scale detection method for '*Ca. L. asiaticus*' using sensitive non-radioactive PCR probes and tissue blots on nylon membranes. The objectives of this paper are to (i) identify and develop non-radioactive DNA probes for the detection of '*Ca. L. asiaticus*', and (ii) develop a tissue blot hybridization method for the rapid and large scale detection of '*Ca. L. asiaticus*' from the field samples.

2. Materials and methods

2.1. Plant materials

To study the citrus '*Ca. L. asiaticus*' bacterium, young healthy citrus plants were graft inoculated with budwood from '*Ca. L. asiaticus*'-infected citrus trees [8] from the field, and kept at the Citrus Research and Education Center (CREC), University of Florida, Lake Alfred in a United States Department of Agriculture Animal and Plant Health Inspection Service/Center for Disease Control (USDA-APHIS/CDC)-approved secured greenhouse at 28 °C (Fig. 1). All young citrus plants used in the greenhouse experiments were HLB-free before graft inoculation, based on PCR and real-time PCR tests. '*Ca. L. asiaticus*'-infected leaves and bark, both young and mature, were collected from symptomatic and asymptomatic branches from greenhouse raised plants as well as directly from the citrus groves and used for direct tissue blotting experiments. For comparison, the healthy citrus tissue was also collected from HLB free greenhouse plants.

2.2. Psyllid samples

Healthy as well as '*Ca. L. asiaticus*'-infected psyllids and nymphs were collected from USDA-APHIS/CDC-approved secured greenhouse at CREC and used for the extraction of total nucleic acids and psyllid squash blots.

2.3. Extraction of total DNA

Total DNA was extracted from 100 mg of tissue (leaf and/or inner bark tissue from healthy as well as '*Ca. L. asiaticus*'-infected plants), and ground in liquid nitrogen, transferred to an eppendorf tube, 1.0 ml of buffer (50 mM Tris–HCl, pH 9.0; 0.1 M NaCl; 10 mM EDTA; and 2% SDS) was added, mixed thoroughly and incubated at 65 °C for 30 min, followed by two phenol:chloroform extractions. Total DNA from 200 μl of the aqueous phase was precipitated and the pellets were suspended in 100 μl of water. Adult psyllids and nymphs were collected and kept at −20 °C for at least 20 min prior to total DNA extraction using Qiagen DNeasy Blood and Tissue Kit (Qiagen, USA) according to the manufacturer's instructions.

2.4. Polymerase chain reaction (PCR)

Based on *Ca. L. asiaticus*' genome sequence [15]; GenBank accession number NZ_ABQW01000001 to NZ_ABQW01000034, total of 34 contigs], 30 gene specific primer pairs for the t-RNA methyltransferase, elongation factor (EF-TU) proteins, outer membrane protein (OMP), RNA polymerase β-subunit, DNA polymerase region, the rDNA region, and the 23S and 16S ribosomal RNA intergenic regions etc., were developed (PrimerQuest^{SM}-Integrated DNA Technologies, USA). These primers were used for the amplification of '*Ca. L. asiaticus*'-specific targets in the '*Ca. L. asiaticus*'-infected citrus plants. In addition, primer pairs for several putative effector genes (CLIBASIA; Prophage related gene clusters) that probably are involved in HLB pathogenesis were also used. These effector genes are highly specific to *Ca. L. asiaticus* and putatively encode proteins which have not been annotated to any specific function.

PCR with SpeedSTAR HS DNA polymerase (Takara Bio, WI) was used to examine the presence of the '*Ca. L. asiaticus*' pathogen in citrus tissues. The PCR (20 μl volume) consisted of 1 μl of DNA template, 0.2 μM each oligonucleotide (gene specific Forward and Reverse primers; Table 1), 0.25 mM dNTPs, 1× buffer (FBII; Takara Bio, USA), and 0.125 μl (5 U/μl) of SpeedSTAR HS DNA polymerase by using the following protocol (primer pairs 1–9, Table 1): 94 °C for 2 min; followed by 10 cycles at 94 °C for 10 s, 54 °C for 10 s, and 72 °C for 90 s; followed by 25 cycles at 94 °C for 10 s, 58 °C for 10 s, and 72 °C for 90 s; followed by final extension at 72 °C for 5 min. For primer pairs 10–18 (Table 1), the PCR cycle was modified slightly with the extension being carried out for 60 s instead of 90 s. PCR reaction (15 μl) was analyzed through 1.4% agarose gel in 1× Tris–acetate–EDTA buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.5) and DNA bands were visualized by ethidium bromide staining. The PCR products were eluted from the gel and purified

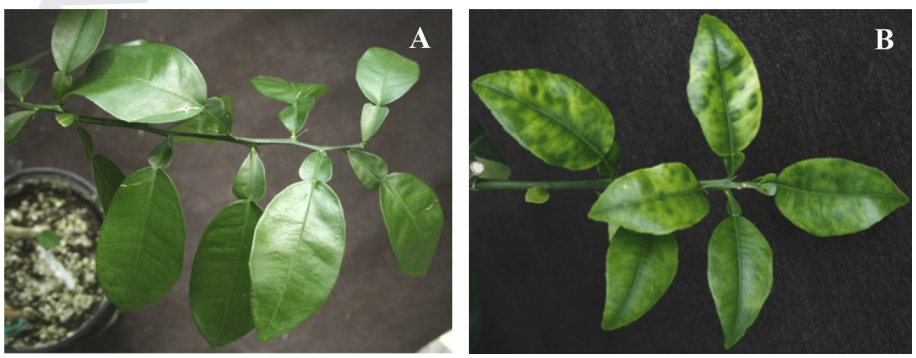

Fig. 1. A, Healthy citrus plant, B, citrus plant infected with '*Ca. L. asiaticus*' showing blotchy mottled symptoms.

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Table 1
Sequence of forward and reverse primers used to prepare DIG-labeled probes for tissue blot experiments.

Serial no.	Primer name	GenBank entry	Gene target	Primer sequence (5′–3′)	Amplicon size
1	HLB135 HLB136	EF164805.1	tRNA methyl RNA protein chain elongation factor EF-Tu (tufB)	F: TACTAATACGACTCACTATAGGGCTTGTTGTGCAGGGCAAGATGTGT R: CCAGATGCAACGCCAACTTCACTT	1 kb
2	HLB141 HLB142	EF164805.1	tRNA methyl RNA protein chain elongation factor EF-Tu (tufB)	F: AATCCTTGTGTCGAGGGTTCAAATCCC R: TACTAATACGACTCACTATAGGGACTATATACC	2.5 kb
3	HLB157 HLB158	JF811345.1	Outer membrane protein	F: CACCGTAGAAGGGCATATTGAT R: CATGCGATTACCTATACGAAAACC	1.3 kb
4	HLB159 HLB160	FJ489642.1	Outer membrane protein	F: CACCATGATAGTTCCTATTACTG R: AAACTGCTGAATTTTATCACCCTC	900 bp
5	HLB163 HLB164	AB473570.1	RNA polymerase beta subunit	F: GCGTTCATGTAGAAGTTGTG R: CCTACAGGTGGCTGACTCAT	500 bp
6	HLB175 HLB176	CP001677.4	ATP-binding protein; Prophage related gene cluster-1	F: CCTTAATACGACTCACTATAGGCGCGTATGCAATACGAGCGGCA R: CGAGACCCGGGAAATACACAAAATACAGCTAAAAATCACTG	1.5 kb
7	HLB177 HLB179	CP001677.4	Prophage related gene cluster-1, elongation factor EF-Tu	F: CTGATATTGTGAACCATGGGAACATCCTCAC R: GATAGACCAAACAGATCAGCGACAACACCGACGGC	1.5 kb
8	HLB178 HLB179	CP001677.4	Prophage related gene cluster-1, elongation factor EF-Tu	F: GTGAGGATGTTCCCATGGTTCACAATATCAG R: GATAGACCAAACAGATCAGCGACAACACCGACGGC	825 bp
9	HLB180 HLB181	CP001677.4	Transcriptional regulator	F: GCCGTCGGTGTTGTCGCTGATCTGTTTGGTCTATC R: AACGGTTAGTTCACGCTAGAGGATATACTAAAACGG	500 bp
10	CL165	CLIBASIA_05165	Hypothetical protein	F: GACCTTAATTAATGGATCAAAAGCAACAAGCATTCCATG R: TTATTGTGCAGTGTTCTTTTTCTGTTTTGTCTG	480 bp
11	CL195	CLIBASIA_05195	Hypothetical protein	F: GAGCTTAATTAATGAACGTGCTAGCCATATTAAATACCGTTTG R: TTATCCTCCTAGCCAAAGGATCTTTTCCGC	615 bp
12	CL200	CLIBASIA_05200	Hypothetical protein	F: GAGCTTAATTAATGGCAAAAGATTTCGCTTCTTTATTAATTG R: CTACCACGAACGGATATCATTCTGCTGTAAT	564 bp
13	CL605	CLIBASIA_05605	Hypothetical protein	F: GAGCTTAATTTATGAGTTTCTTAGATTCGAGTGTAAAATTCGGTTC R: TCATCGTACATGCACCCCTGATAAAAGACTCG	360 bp
14	CL620	CLIBASIA_05620	Hypothetical protein	F: GAGCTTAATTAAATGTACGCTCACAAATACACAAAAGAAAGAATTG R: TTAGTCATGTTTAGTTAACTTCGGAAGCTCAAG	500 bp
15	CL635	CLIBASIA_05635	Hypothetical protein	F: GAGCTTAATTAATGGGACAATTAAAGCAATATTACCTCGAAGAG R: TCAAATCTGACTGGCGTAATAATCTTCTTTATG	420 bp
16	CL665	CLIBASIA_05665	Hypothetical protein	F: GAGCTTAATTAATGGGAAGAAAAGTTTTAACACCTGAAGAAAGG R: TTAACCTCTCTTATAAATTAGTTCTTCTATGG	417 bp
17	OMP1	CLIBASIA_00995	Outer membrane protein	F: TGCAGGCTCGGTACCACAAACTAA R: TGCATCATAGCCACCTGTCACTGT	475 bp
18	OMP2	CLIBASIA_02425	Outer membrane protein	F: ACTCGCCTCTTTCTGTTCTGCTC R: AATCCTCCGACTTCAACACCGACA	474 bp

using GeneClean® Kit (MP Biomedicals, USA) following the manufacturer's instructions.

2.5. Digoxigenin labeling and probe preparation

Digoxigenin-11-dUTP (DIG-11-dUTP) probes were synthesized by PCR amplification of the purified DNA (PCR products) in 50 μl reaction volume using the PCR DIG-Probe Synthesis Kit (Roche, USA) following the manufacturer's instructions. Reactions consisted of a denaturation step of 2 min at 94 °C, 35 cycles of 10 s at 94 °C, 10 s at 58 °C and 90 s at 72 °C and a final step extension of 3 min at 72 °C. PCR products were analyzed in 1.4% agarose gels. The probes were denatured at 70 °C for 10 min, centrifuged at 10,000 rpm for 5 min, then dissolved in 25 ml of pre-hybridization buffer (300 mM $C_6H_5Na_3O_7$, 3 M NaCl, 10% SDS, 50% formamide, pH 7.5) and used for the detection of HLB-specific targets in hybridization experiments.

2.6. Membrane preparation, hybridization and detection

The blotted nylon membranes (positively charged nylon membranes, Roche, USA), were kept in the denaturation solution (1.5 M NaCl, 0.5 N NaOH) for 10 min, transferred to neutralization solution (0.5 N HCl, 0.5 M NaCl) for 10 min, and then washed with 2× SSC by shaking for 2 min. The membranes were irradiated with 1.2×10^5 μJ UV light over 30 s in a UV cross-linker (Stratalinker, Sratagene, USA) and stored at 4 °C or used immediately. The hybridizations were carried out in a Techne Hybridiser HB-1D (Bibby Scientific, USA) hybridization oven. The membranes were pre-hybridized in pre-hybridization buffer at 42 °C for 1 h. The pre-hybridization buffer was replaced with 25 ml of fresh hybridization buffer containing 50 ng/ml DIG-labeled probe (pre-heat at 68 °C for 10 min just before use) and the membranes were incubated at 42 °C for 16–20 h. The membranes were washed twice with 2× SSC, 0.1% SDS at RT for 5 min each, twice with 0.1× SSC, 0.1% SDS at 68 °C for 15 min each. The membranes were further washed once with maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 2 min, blocked in maleic acid buffer containing 1× blocking solution (Roche, USA) for 30 min at RT and incubated with "Anti-Digoxigenin AP Anti Fab fragment" (DIG antibody, 75 mU/ml, Roche, USA) in blocking solution at RT for 30 min. The membranes were washed twice with washing buffer (maleic acid buffer containing 0.3% Tween20) for 15 min each followed by incubation in detection buffer (0.1 M Tris–HCl, 0.1 M NaCl, pH 9.5) for 5 min. The membrane was placed between clean plastic sheets and 'CDP-Star' (Roche, USA, 1 ml for every 100 cm^2 of the blot) was added. The membrane was then sealed and incubated in dark at RT. After 5 min the excess liquid was squeezed out of the membrane bag and was exposed to the X-ray film (Kodak film, Carestream Health Inc., USA).

2.7. Dot blot hybridization using serial dilutions

Three dilutions of the total DNA extracted from plant and psyllid ('*Ca. L. asiaticus*' positive and control) samples (0.1, 0.01 and 0.001) were applied onto the positively charged nylon membrane using a Dot blot apparatus (Bethesda Research Laboratories, USA). Membrane preparation, pre-hybridization, hybridization, post-hybridization washes, detection were same as mentioned above. Elongation factor (EF-TU amplified using primer pair 2; Table 1)

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cloned in the transcription vector, pGEM-T, was used as a positive control in dot-blot hybridizations (Fig. 2).

2.8. Tissue blot preparation

Citrus tissues such as leaves, petioles, thorns, inner bark of the twigs, cross-section of the twig, apical meristems, midribs, floral petals, pistils, stamens, nymphs, psyllids, etc., were used for tissue blot/squash screening. Leaf samples were rolled into a tight bundle, sliced with a razor blade and the freshly cut leaf region was blotted on to nylon membrane by gently pressing and holding for 3–5 s against the membrane. The stems/petiole pieces were washed thoroughly with sterile distilled water, cut obliquely (to increase the surface area) and the cut surface was pressed onto the membrane. A small twig with 5–7 leaves was gently scraped with a razor blade, changing blades for each leaf and stem of the twig, and the scraped surface was gently pressed and held onto the membrane. Total DNA and crude extract of nymphs and psyllids was also loaded onto the membrane. Nymphs and psyllids were directly squashed onto the membrane.

2.9. Real-time PCR

The gene specific (Table 2) real-time primer pairs were developed using IDTSciTools-RealTimePCR, Integrated DNA Technologies, USA. Real-time PCR was performed using a 96-well optical reaction plate (Applied Biosystems, USA) in the ABI Prism 7500 Real-Time-PCR System (Applied Biosystems, USA). Each well contained a 20 μl reaction mixture containing 10 μl of 2× SYBR Green PCR Master Mix (RT2 Real-Time™ SYBR Green/Rox PCR, SABiosciences, USA) and 2 μl primer (0.2 μM each of forward and reverse primer), 2 μl target DNA (‘*Ca. L. asiaticus*’ positive and/or control). The PCR was performed using the following conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles each of 95 °C for 15 s, 60 °C for 1 min. Melting curve reaction was performed at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 30 s at the rate of 0.2 °C. A standard curve was developed using a series of dilutions of the target DNA (10^2–10^7). Internal control included COX primers (F: 5′-GTATGCCACGTCGCATTCCAGA-3′; R: 5′-GAATGCCCTTAGCAGTTTTGGC-3′) with healthy citrus DNA sample [14]. All reactions were performed in triplicate with positive, healthy and water controls, and the mean

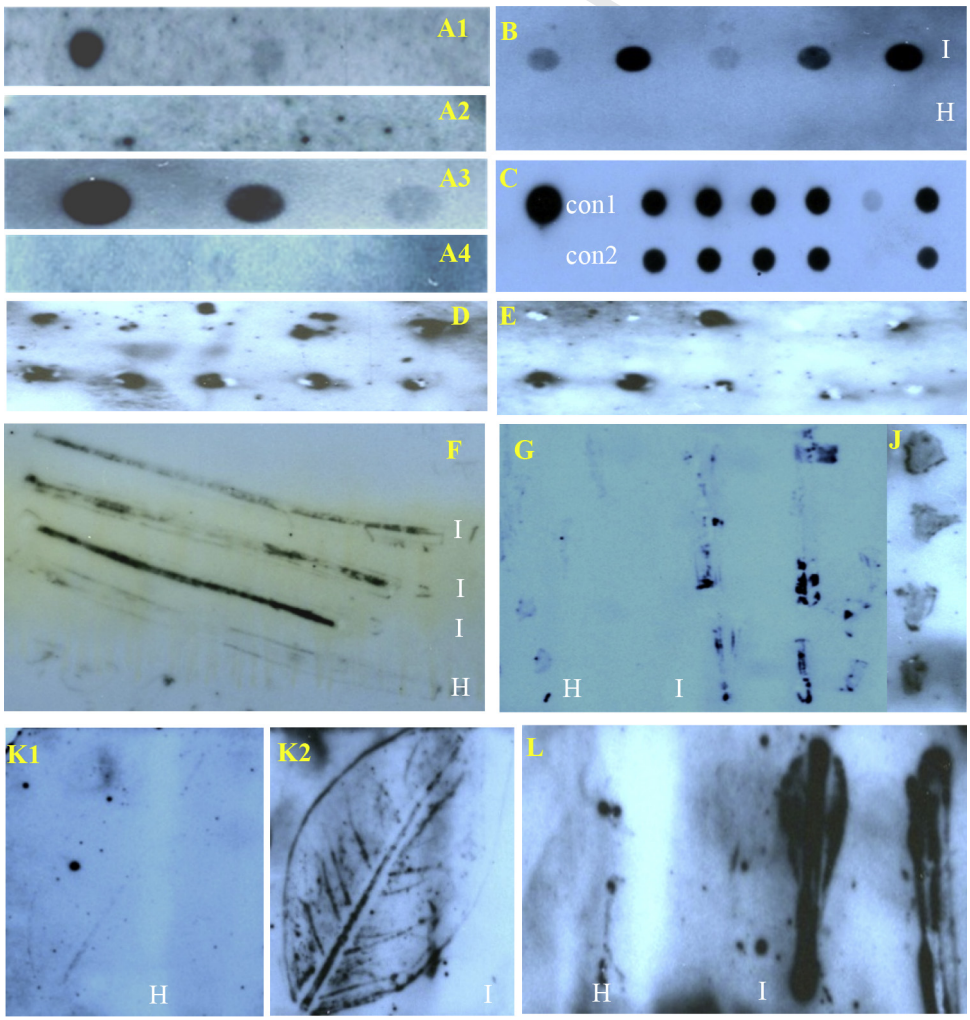

Fig. 2. Dot-blot of serially diluted total nucleic acid extracts from A1, ‘*Ca. L. asiaticus*’-infected psyllid and A2, healthy psyllid sample, A3, ‘*Ca. L. asiaticus*’-infected, and A4, healthy plant sample using Probe 4, B, dot-blot from healthy [H] and ‘*Ca. L. asiaticus*’-infected [I] leaf samples using Probe 9, C, crude extract blot of ‘*Ca. L. asiaticus*’-infected nymphs. Con1 is control (EFTU fragment cloned into poem-T vector and con2 is water sample using Probe 2, D, squash-blot of ‘*Ca. L. asiaticus*’-infected nymphs using Probe 2, E, squash-blot of ‘*Ca. L. asiaticus*’-infected Psyllids using Probe 7, F, tissue blot of inner bark of twigs of healthy [H] and ‘*Ca. L. asiaticus*’-infected [I] samples using Probe 4, G, tissue blot of leaf mid-ribs of healthy [H] and ‘*Ca. L. asiaticus*’-infected [I] samples using Probe 9, J, bark cut diagonally and imprinted on the membrane using Probe 7, K, leaf of healthy [H; K1] and ‘*Ca. L. asiaticus*’-infected [I; K2] tissue-blots using Probe 7, L, petiole and bark of healthy [H] and ‘*Ca. L. asiaticus*’-infected [I] tissue blot using Probe 2.

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Table 2
Sequence of forward and reverse primers used for real-time PCR experiment.

Serial no.	Primer name	GenBank entry	Gene target	Primer sequence (5′–3′)	Amplicon size
1	HLB141 HLB142	EF164805.1	tRNA methyl RNA protein chain elongation factor EF-Tu (tufB)	F: AATCCTTGTGTCGAGGGTTCAAATCCC R: TACTAATACGACTCACTATAGGGACTATATACC	2.5 kb
2	HLB178 HLB179	CP001677.4	Prophage related gene cluster-1, 2elongation factor EF-Tu	F: GTGAGGATGTTCCCATGGTTCACAATATCAG R: GATAGACCAAACAGATCAGCGACAACACCGACGGC	825 bp
3	CL165	CLIBASIA_05165	Hypothetical protein	F: GACCTTAATTAATGGATCAAAAGCAACAAGCATTCCATG R: TTATTGTGCAGTGTTCTTTTTCTGTTTTGTCTG	480 bp
4	CL200	CLIBASIA_05200	Hypothetical protein	F: GAGCTTAATTAATGGCAAAAGATTTCGCTTCTTTATTAATTG R: CTACCACGAACGGATATCATTCTGCTGTAAT	564 bp
5	CL605	CLIBASIA_05605	Hypothetical protein	F: GAGCTTAATTTATGAGTTTCTTAGATTCGAGTGTAAAATTCGGTTC R: TCATCGTACATGCACCCCTGATAAAAGACTCG	360 bp
6	CL620	CLIBASIA_05620	Hypothetical protein	F: GAGCTTAATTAAATGTACGCTCACAAATACACAAAAGAAAGAATTG R: TTAGTCATGTTTAGTTAACTTCGGAAGCTCAAG	500 bp

value of the threshold cycle (Ct) was presented with standard deviation.

3. Results and discussion

To develop a viable management strategy for HLB disease in citrus there is a greater need to develop rapid detection methods which includes early diagnosis for the presence of the pathogen in citrus in the field. Early detection in the field on large-scale using tissue blot technology is one of the viable strategies. The primer pairs developed in this study were specific to '*Ca. L. asiaticus*' pathogen and they did not show any cross genome amplification in '*Ca. L. africanus*' and '*Ca. L. americanus*' [16]. The specificity of these primers was also tested in silico using BLASTn search. No sequence matched except for the query sequence, suggesting that these primers were specific to '*Ca. L. asiaticus*'.

Of the 30 primer pairs, 18 primer pairs (Table 1) amplified specific amplicons from the DNA isolated from '*Ca. L. asiaticus*'-infected plants and no products were amplified from the healthy plants. We used these primer pairs to prepare non-radioactive Digoxigenin (DIG) labeled DNA probes. Labeling efficiency of the probes was determined using a series of sample dilutions. The probes were able to detect as little as 0.001 dilutions of '*Ca. L. asiaticus*'-infected citrus plant DNA and the psyllid DNA in the dot blot hybridization experiments (Fig. 2). Although the dot blot hybridization technique required additional time for sample preparation, it allowed the detection/quantification of the sensitivity and specificity of the DIG probes. Similar study was also reported in tomato using total DNA extracted from fresh leaves [17]. This technique was also useful in quantification of the '*Ca. L. asiaticus*' in individual psyllids. A linear relationship was observed between the signal and the amounts of the DNA used in the dot blots. Quantification of tomato yellow leaf curl gemini virus DNA in individual whiteflies using dot blot has also been reported [18]. The dot blots clearly showed positive hybridization signals for the '*Ca. L. asiaticus*'-infected citrus, psyllid and nymph DNA and no cross-reaction was observed with control healthy and water samples (Fig. 2).

To detect the presence of '*Ca. L. asiaticus*', in the present study, different tissue parts of citrus trees were examined by direct tissue blots for the presence of the pathogen. Direct tissue blots ('*Ca. L. asiaticus*'-infected and healthy plants as well as '*Ca. L. asiaticus*'-infected and healthy psyllids and nymphs) on nylon membranes were used as targets for nucleic acid hybridization experiments. The '*Ca. L. asiaticus*' bacterium was readily detected in all the tissue blots (leaves, petioles, thorns, inner bark of the twigs, cross-section of the twig, apical meristems, midribs, floral petals, pistils, stamens and peduncles). However, higher concentration of '*Ca. L. asiaticus*' bacterium was found in inner bark of the twigs, midribs, petioles, ovaries and peduncles of '*Ca. L. asiaticus*'-infected plants (Fig. 2), whereas the healthy plants had no '*Ca. L. asiaticus*'-specific DNA hybridization. The results were confirmed/reconfirmed with different probes (Table 1) using at least 6–8 membrane blots for each tissue blot (both from field as well as greenhouse samples). Each membrane had 20–25 insect samples or 5–8 plant samples and the probes developed for primer pairs 2 and 8 gave the best early detection signals. In each case non-uniform hybridization probably suggested uneven distribution of '*Ca. L. asiaticus*' bacterium within the host plant. A 2.6 kb ^{32}P-labeled probe was used to detect the Asian strains of HLB bacterium in the leaves exhibiting the blotchy mottle symptoms in Inida by DNA–DNA hybridization [19]. The sensitivity of DIG labeled probes was also tested using In 1.7 and AS 1.7 fragments of *Ca. L. asiaticus* and *Ca. L. africanus* respectively [20]. Both these studies required the extraction of DNA from the samples collected for HLB detection. Of recent, 0.5 mm^2 pieces of direct tissue blots were treated with distilled water or glycine buffer and the extract was used to carry out real-time PCR for the detection of all *Ca. Liberibacter* species [21]. Direct tissue immuno-blots have been used successfully for the detection of many plant viruses [22–28]. Tissue blot hybridizations for the detection of viroids in potato and pome fruits with chemiluminescent detection using DIG labeled probes have shown that the hybridizations were as sensitive as autoradiography using ^{32}P probes [29,30], and with apple scar skin viroid in pear [31] it was shown that the detection was much faster with tissue blot hybridizations.

Higher concentration of '*Ca. L. asiaticus*' bacterium has also been detected in bark tissues, leaf midribs and different floral and fruit parts using PCR and real-time PCR [10] as was observed in the present study using DIG labeled DNA probes. Though PCR assays were accepted as confirmatory tests for the detection of HLB in host plants or psyllids, the poor amplification from even highly suspicious trees with severe symptoms [10,14,32] led to the development of new PCR primers from the 16S rDNA sequences of three CLIBASIA genes of '*Ca. L. asiaticus*' which had higher sensitivity as compared to the other commonly used PCR sets [33]. A number of real-time PCR protocols have been developed for the detection of HLB [10,14,32,34]. Of recent, real-time PCR using nearly identical tandem-repeats of two '*Ca. L. asiaticus*' prophage genes was utilized for the detection of '*Ca. L. asiaticus*' [35]. Both these recent studies reported the identification of HLB from various tissues/samples, however, the impracticality of utilizing conventional and real-time PCR for screening large number of samples in the field, without DNA extraction, warrants the need of new techniques for the detection of HLB such as the one presented in this study.

Albrecht and Bowman [36] also observed high concentration of HLB bacterium in peduncles. Molecular hybridization of tissue-

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blotted membranes can be successfully used for the detection of bacterium avoiding the need to process the samples. Tissue blotting reduces test times by several hours to as much as a day and eliminates the need for phenol/chloroform extractions [30,37] as seen in the present study. There was no significant loss in the sensitivity (data not shown) of the DIG labeled DNA probes as they could be used repeatedly up to 12–15 hybridizations without loss of sensitivity. DIG labeled DNA probes are cheap to produce, stable upon storage, easy to handle, could be used with ease without obvious risks of using radioactivity [30].

The detection of '*Ca. L. asiaticus*' bacterium in psyllids and/or nymphs by tissue blotting (squash blot) on the nylon membrane was also carried out. However, background signal was observed due to various body parts (wings, legs, hair etc.) of psyllids and nymphs sticking to nylon membrane during squashing process, and was very hard to exclude during membrane wash. To avoid background hybridization signals, pretreatment of membranes with 20× SSC before squash [30] and treatment of imprinted membranes with 2M mercaptoethnol for 10 min and rinsing twice with water before pre-hybridization [38] was attempted. These steps did not affect the sensitivity. Separating the head, legs and wings from the abdomen region before tissue blotting, helped to reduce the membrane background. Use of crude extracts (10 μl of the supernatant) of psyllids instead of whole psyllids substantially helped in reducing the background. Compared to adult psyllids, higher concentration of '*Ca. L. asiaticus*' bacterium was found in nymphs (Fig. 2) as has been observed previously [9,39]. Squash blot hybridization of psyllids using ^{32}P-labeled probe has been reported [19].

In the HLB infected trees, the bacterium is distributed unevenly and often in an erratic manner even within the same tree. Sometimes some branches show the symptoms while the adjoining branches almost look healthy with low concentration of the bacterium below the detection threshold [10,40]. Different citrus cultivars become infected with HLB to varying extent under greenhouse conditions [8]. Graft inoculations of the healthy citrus under greenhouse conditions with '*Ca. L. asiaticus*'-infected buds from the '*Ca. L. asiaticus*' positive field trees induce symptoms and accumulate bacterium at different levels [8]. In an effort to understand the nature of distribution, a branch from a '*Ca. L. asiaticus*'-infected citrus plant with symptomatic and non-symptomatic leaves was used for tissue blotting and hybridization with DIG labeled EF-TU probe. The DIG labeled probes developed in this study could effectively detect lower concentrations of '*Ca. L. asiaticus*' bacterium even in the non-symptomatic leaves, within the same branch, that are otherwise difficult to detect visually from plants both from the greenhouse as well as from the field (Fig. 3). We also tested whether the age of the tissue has an effect on the detection of '*Ca. L. asiaticus*' bacterium using a young and old branch tissue samples from greenhouse as well as from the field. The age of the tissue did not have any effect on the detection of the '*Ca. L. asiaticus*' bacterium by tissue blotting as imprints made by both young and old branch tissues gave similar hybridization signals (Fig. 3). However, the older tissues were much easier to imprint

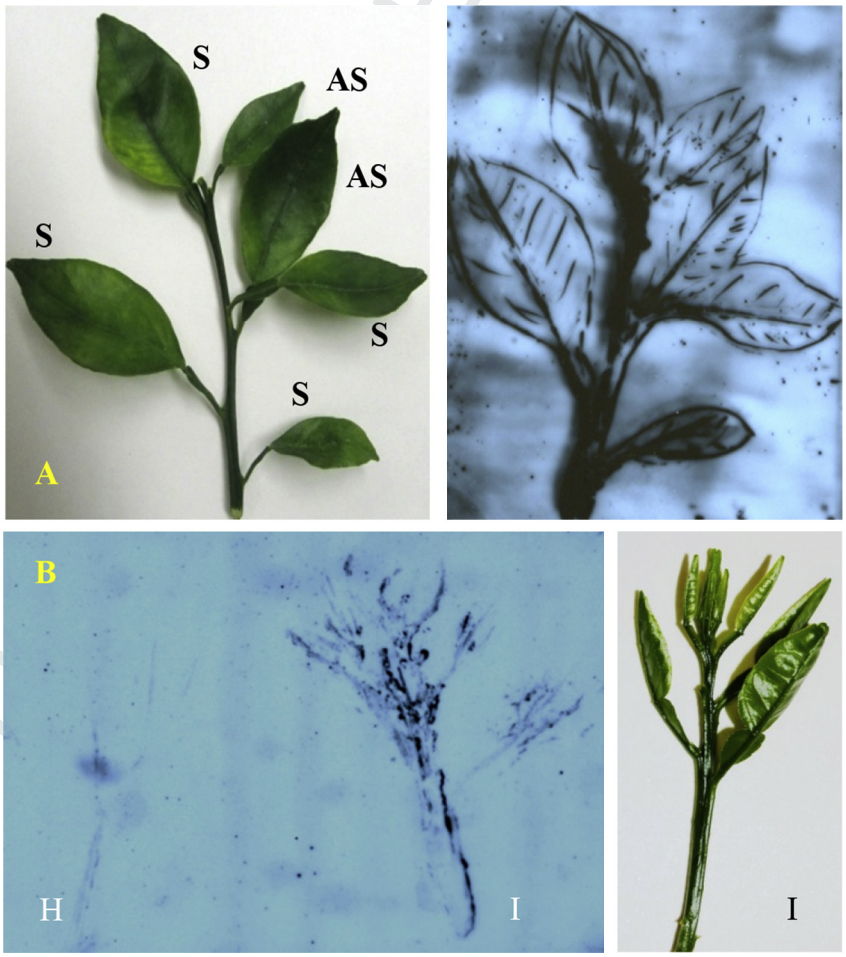

Fig. 3. Old (A) and young branch (B) from a '*Ca. L. asiaticus*'-infected citrus plant with symptomatic (S) and non-symptomatic (AS) leaves used for tissue-blotting and hybridization with DIG labeled probe 2 specific for '*Ca. L. asiaticus*'. [H] Healthy and '*Ca. L. asiaticus*'-infected [I] samples.

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on the nylon membrane and facilitated easy identification of positive hybridization signals.

The hybridization signals obtained with other CLIBASIA probes (Table 1; primer pairs 10–18) were weak as compared to the EF-TU and other probes (Table 1; primer pairs 1–9) with all the citrus tissue samples, as well as psyllid and nymph samples tested (data not presented). To confirm if the weak hybridization signal is due to low copy number of the target genes, we performed real-time PCR experiments with EF-TU and CLIBASIA primer pairs (Table 2). The real-time PCR protocol allowed positive '*Ca. L. asiaticus*' detection in citrus samples infected with '*Ca. L. asiaticus*' bacterium. The first derivative of the melting curve showed single peak in all cases (Fig. 4) further confirming the absence of significant primer–dimer or nonspecific amplification products. The *Tm* values obtained for EF-TU primer pairs were 79.50 °C–79.69 °C, where as those from CLIBASIA primer pairs were 75.8 °C–79.5 °C. The amplification products were analyzed by 2% agarose gel electrophoresis and in all cases a single band of the expected size was observed with no primer–dimer formation. No amplification was observed with either negative control including healthy plant extract or with water alone, confirming the specificity of the assay. To calculate the number of gDNA copies, standard curves were prepared using 10-fold serial dilutions of target DNA samples from '*Ca. L. asiaticus*'-infected plants. Standard curves showed a strong linear relationship with correlation coefficients of 0.997 for EF-TU probes and 0.999 for CLIBASIA primer pairs (Fig. 5), and their amplification efficiencies were over 90%. The average Ct values were within the dynamic range of the standard curve and 18.52 ± 0.08 for EF-TU primer pairs and that for CLIBASIA primer pairs were 22.21 ± 0.05. The coefficient of variation values were very low 0.004 between assays, indicating good reproducibility of the technique.

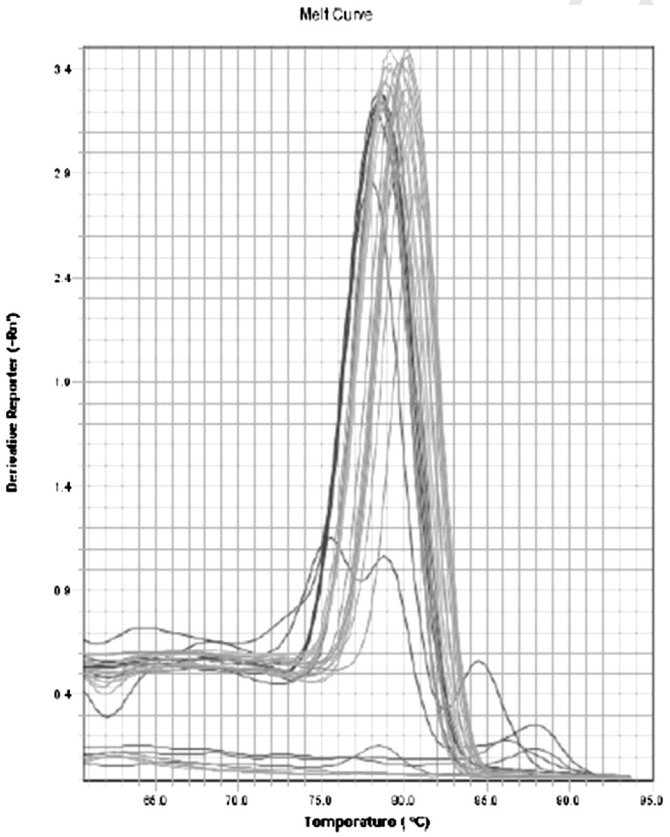

Fig. 4. A melt curve obtained in real-time PCR with Clibasia-4 primer pair (Table 2) for '*Ca. L. asiaticus*' positive citrus leaf sample.

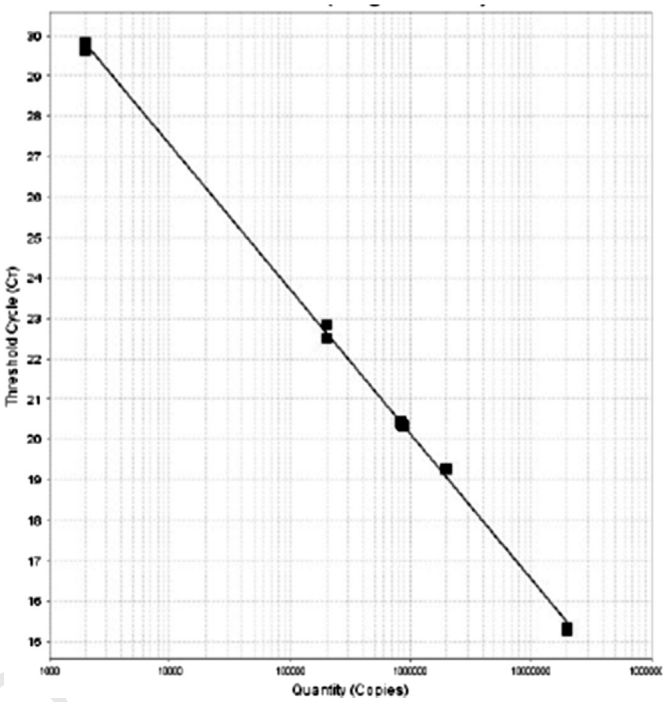

Fig. 5. Standard curves and linear regression ($R^2 = 0.999$) obtained in real-time PCR with Clibasia-4 primer pair (Table 2) for '*Ca. L. asiaticus*' positive citrus leaf sample.

The estimated number of gDNA copies for the EF-TU primers pairs were $2.5 \times 10^6 \pm 1.2 \times 10^5$ and that for CLIBASIA primer pairs were $2.8 \times 10^5 \pm 8.7 \times 10^3$. Our results confirm the weak signal obtained with CLIBASIA probes probably represented low copy number of the target genes in the '*Ca. L. asiaticus*' genome. Similar *C*t values between the ranges of 11.20 + 1.02 to 30.48 + 1.67 [35], 20.96 to 26.32 [34] and 21.2 to 24.3 [41] have been reported for the detection of '*Ca. L. asiaticus*' in citrus and psyllids. The correlation coefficients of 0.997 and 0.996 were also reported by Li et al. [34] and Teixeira et al. [41], respectively.

In conclusion, the tissue blot technique, potentially, is the method of choice for processing many plant/psyllid samples on a single piece of membrane; it is easy, rapid, reliable, non-radioactive and can identify infected plants before symptom appearance. Using this technique, large number of samples could be analyzed with least amount of ambiguity and could be especially useful to test infection ratios in populations of psyllids. Tissue imprints, once on the membrane could be stored for later use, can be shipped easily and generally are stable. The blots can be made on site and does not involve live tissue storage and transportation. The modified form of tissue blot, the 'squash-blot', for psyllids would be useful to understand the epidemiology of '*Ca. L. asiaticus*' bacterium.

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