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Quantitative real-time PCR for detection and identification of *Candidatus Liberibacter* species associated with citrus huanglongbing

Wenbin Li [a], John S. Hartung [b], Laurene Levy [a,*]

[a] *National Plant Germplasm and Biotechnology Laboratory, USDA-APHIS-PPQ-CPHST, Beltsville, MD 20705, USA*
[b] *Fruit Laboratory, USDA-ARS,10300 Baltimore Avenue, Beltsville, MD 20705, USA*

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Abstract

Citrus huanglongbing (HLB, ex greening) is one of the most serious diseases of citrus. Different forms of the disease are caused by different *Candidatus Liberobacter* species, *Candidatus Liberibacter asiaticus* (Las), *Ca. L. africanus* (Laf) and *Ca. L. americanus* (Lam). The pathogen is transmitted by psyllid insects and by budding with contaminated plant materials. The vector psyllid *Diaphorina citri* can transmit both Las and Lam. Establishment of this vector into Florida, reports of Lam and Las in Brazil in 2004, and recent confirmation of HLB in Florida in September 2005 is of great concern to the citrus industry. Research on HLB has been hampered by the unculturable nature of the causal bacterium in artificial media. It has also been difficult to detect and identify the pathogens, possibly because of low concentration and uneven distribution in host plants and vector psyllids. In this study, we developed quantitative TaqMan PCR using 16S rDNA-based TaqMan primer–probe sets specific to the different *Ca. Liberobacter* spp. An additional primer–probe set based on plant cytochrome oxidase (COX) was used as a positive internal control to assess the quality of the DNA extracts. The assays do not cross-react with other pathogens or endophytes commonly resident in citrus plants, and are very sensitive. HLB pathogen DNA was successfully amplified from the equivalent of 20 ng of midrib tissue from symptomatic leaves. The consistent results of the assays with DNA extracted from plants infected by various *Ca. Liberibacter* species grown in greenhouses and in the field demonstrated a degree of reproducibility for these TaqMan assays. Inhibitors of the PCR that are frequently present in plant extracts did not affect the assay results. The population of the pathogens was estimated to be 5×10^7 and 2×10^6 cells/g of fresh midribs of symptomatic sweet orange leaves infected by Las and Lam, respectively. The ratio of pathogen DNA to host plant DNA was estimated by to be 1:13,000 (w/w) and 1:1000 (c/c: target copy/target copy) in DNA extracts obtained by a standard CTAB method. Our rapid, sensitive and specific TaqMan PCR assay for the detection, identification and quantification of *Ca. Liberibacter* species has been successfully used in the confirmation of HLB caused by Las in Florida, and will be very useful for a broad range of research programs as well as the regulatory response and management of HLB disease. Published by Elsevier B.V.

Keywords: Molecular diagnosis; Citrus greening; Phloem-limited bacterium; HLB

1. Introduction

Citrus huanglongbing (HLB), ex citrus greening (da Graça and Korsten, 2004), is probably the most seri-

* Corresponding author.
E-mail address: Laurene.Levy@aphis.usda.gov (L. Levy).

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ous disease of citrus caused by an insect-vectored pathogen (Halbert and Manjunath, 2004). The disease is wide spread in Asian countries, the Indian Subcontinent and Ocean, southern Africa and recently in Brazil (da Graça, 1991; da Graça and Korsten, 2004; Halbert and Manjunath, 2004; Coletta-Filho et al., 2004; Teixeira et al., 2005a). The causal agent is a fastidious prokaryote that has not yet been cultured, lives in the sieve tube elements of infected plants, is systemic in infected insects and is taxonomically in the α-subdivision of the *Proteobacteria* (Jagoueix et al., 1994). HLB exists in nature in three forms that differ by a combination of environmental conditions and insect vectors. HLB caused by *Candidatus Liberibacter asiaticus* (Las) is a heat-tolerant form vectored by *Diaphorina citri*. HLB caused by *Ca. L. africanus* (Laf) is a heat-sensitive form and vectored by *Trioza erytreae*. HLB caused by *Ca. L. americanus* (Lam) is a heat-tolerant form vectored by *D. citri*. These forms of HLB occur respectively in Asia, southern Africa and Brazil (Jagoueix et al., 1994; Teixeira et al., 2005a,c). HLB caused by Las was detected and confirmed in Florida by the US Department of Agriculture's Animal and Plant Healthy Inspection Services and the Florida Department of Agriculture and Consumer Services in September 2005 (Knighten et al., 2005). Since the vector psyllid *D. citri* has become established in Florida since its introduction in 1998 (Halbert, 1998), the disease is of great concern to the citrus industry.

D. citri can be moved long distances on leaves, twigs and unprocessed fruit (Halbert and Manjunath, 2004), acquires the pathogens from infected plants in 15–30 min and transmit the pathogens to healthy plants in less than 1 h after a latent period of 8–12 days (Raychaudhuri et al., 1972). It has been difficult to consistently detect the Liberibacters through any of biological assays (Roistacher, 1991), the presence of fluorescent substances used as a marker (Schwarz, 1968), or by using light (Wu, 1987) or electron (Laflèche and Bové, 1970) microscopy, and ELISA (Garnier and Bové, 1993). This is presumably because of the low concentration and the uneven distribution of the pathogens in host plants and vector insects (McClean, 1970; Su and Chang, 1974). In addition, the non-specific nature of foliar symptoms makes the disease difficult to distinguish from nutrient deficiencies or other plant diseases.

Molecular approaches have been used to detect and differentiate *Ca.* species of HLB pathogens. Based on the DNA sequence of the β-operon, two DNA probes were developed for detection of Las and Laf (Villechanoux et al., 1992). However, the sensitivity of dot-blot hybridization assays using these probes had equivalent sensitivity to that of electron microscopy. Conventional PCR methods with specific primers that amplify 16S rDNA sequences have been used to detect Las and Laf (Jagoueix et al., 1996; Tian et al., 1996). Digestion of the PCR products with Xba1 is required to distinguish Las from Laf. A new primer set was developed in 1999 based on ribosomal protein genes of the β-operon to detect and differentiate Las from Laf directly by amplicon size (Hocquellet et al., 1999). An additional primer set specific to Las was developed, based on partial sequence of the β-operon (Hung et al., 1999, 2004). Several months after the first report of HLB in Brazil, two primer sets were developed based on the 16S rDNA sequences to specifically identify Lam, a new *Ca*. species using conventional PCR amplification based on the 16S rDNA sequence (Coletta-Filho, 2005; Teixeira et al., 2005a,b). Recently, loop-mediated isothermal amplification (LAMP) was developed for Las detection in laboratories that lack thermocyclers (Okuda et al., 2005). Although conventional PCR and LAMP methods are sensitive and specific, consistent detection of HLB pathogens in infected plants or vectors remains problematic (Halbert and Manjunath, 2004; Okuda et al., 2005).

Accurate, rapid and robust detection methodologies are needed for a regulatory response and effective management of HLB through the detection and removal of infected trees and development of a *Ca. Liberibacter*-free nursery system. Numerous research studies in HLB disease will also be advanced by the availability of such methods. Real-time, quantitative PCR has gained acceptance due to its improved speed, sensitivity, reproducibility, robustness and the reduced risk of carry-over contamination as compared to standard format PCR (Mackay et al., 2002). We have developed for the first time quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assays using probe-primer sets specific to *Ca. Liberibacter* spp. We have also used a plant cytochrome oxidase (COX)-based primer–probe set as positive internal control to assess the quality of DNA extracts and reaction cocktails. The diagnosis can be performed with DNA extracts in the field in less than 1 h using a portable SmartCycler (Cepheid, Sunnyvale, CA) machine for real-time detection of the pathogens. The assay protocols should only require minimum adaptation for use in an automated ABI system in plant diagnostic laboratories, centers or networks.

2. Materials and methods

2.1. DNA samples

Total DNA was extracted with DNeasy Plant Kits (Qiagen, Valencia, CA) from the midribs (about 200 mg) of three symptomatic or asymptomatic leaves of diseased or healthy greenhouse-grown sweet orange (*Citrus sinensis* L.) trees (about 5 years old) and suspended in 100 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Total DNA was also extracted from 24 subsamples obtained from 4 HLB-symptomatic trees recently discovered in Homestead, Florida using the same method. Total DNA samples kindly provided by Dr. Toru Iwanami (National Agricultural Research Center, Kumamoto, Japan) were obtained using a CTAB (Murray and Thompson, 1980) extraction method from 200 mg of midrib tissue from HLB symptomatic leaves of greenhouse-grown rough lemon (*Citrus jambhiri*) and suspended in 1 ml of TE buffer. DNA samples from Brazil were obtained using CTAB extraction of 500 mg of midrib tissue from symptomatic or asymptomatic leaves of field-grown sweet orange trees and suspended in 100 μl of water. Genomic DNA from pure cultures of *Xanthomonas axonopodis* pv. *citri* (Xac), *Xylella fastidiosa* (Xf) strain L001 (Li et al., 2002), *Phytophthora citricola* I, *Phytophthora citrophthora* I, and the citrus endophytes *Methylobacterium mesophilicum*, *Curtobacterium flaccumfaciens*, *Pantoea agglomerans*, *Enterobacter cloacae* and *Bacillus* sp. (Lacava et al., in press) was extracted using Qiagen kits. Total DNA from citrus plants purposefully infected with *Citrus tristeza* virus (CTV) strain T36, *Citrus* sudden death (CSD) virus or with citrus blight (CB), a disease of unknown etiology, were also extracted using DNeasy kits. The yield and purity of DNA samples were estimated by measuring $OD_{260\ nm}$ and $OD_{260\ nm/280\ nm}$, respectively, with a spectrophotometer SPECTRA max PLUS384 (Molecular Devices, Sunnyvale, CA).

2.2. Primer and probe design

Four primers and one TaqMan probe were empirically designed based on the 16S rDNA sequences of *Ca. Liberibacter* species (GenBank accession numbers: L22532 of Las, L22533 of Laf and AY742824 of Lam) (Fig. 1a). The reverse primer (HLBr) is specific to the genus *Liberibacter* and recognizes all three species (Las, Laf and Lam) in the genus and can be used in combination with the other primers. HLBas, HLBaf and HLBam are primers specific to Las, Laf and Lam, respectively. HLBp, labeled at 5′-terminal nucleotide with 6-carboxy-fluorescein (FAM) reporter dye and at 3′-terminal nucleotide with Black Hole Quencher (BHQ)-1, is the common probe for all TaqMan assays with the primers above. The positive internal control primer–probe set was designed (Fig. 1b) on basis of the sequence (GenBank accession number CX297817) of the conserved plant cytochrome oxidase (COX) gene from citrus in the EST database. The internal probe COX-p was labeled with tetrachloro-6-carboxy-fluorescein (TET) reporter dye at the 5′-terminal nucleotide and with BHQ-2 at the 3′-terminal nucleotide. In silico PCR was performed for all primer–probe combinations by using BLASTn against the NCBI GenBank database to ensure the specificity of the primers and probes prior to synthesis by Integrated DNA Technologies, Inc.

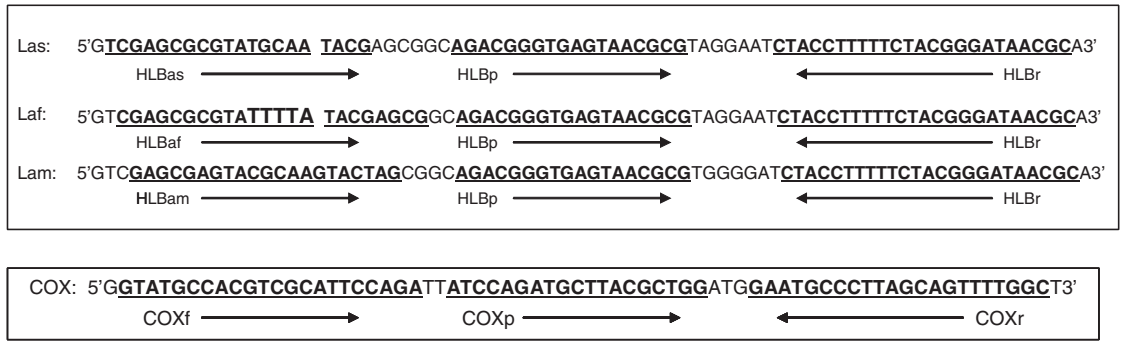

Fig. 1. Sequences of TaqMan primers and probes for amplification of 16S rDNA of *Candidatus* Liberibacter species associated with citrus huanglongbing: (a) primer–probe combinations: HLBaspr is specific to *Ca. L. asiaticus*; HLBafpr specific to *Ca. L. africanus*; HLBampr specific to *Ca. L. americanus*; HLBasampr for mixed infection of *Ca. L. asiaticus* and *Ca. L. americanus*; HLBasafpr for mixed infection of *Ca. L. asiaticus* and *Ca. L. africanus*; COXfpr for positive internal control to verify the quality of DNA extraction and (b) the cytochrome oxidase (COX) gene of host plants for positive internal control.

2.3. Real-time PCR assay

To obtain high endpoint fluorescence and low cycle threshold (Ct) values, optimization of reagents, including concentrations of the target and internal control primers and probes, $MgCl_2$ and dNTPs, and standardization of the protocol including the temperature and the time of denaturation, annealing and amplification were carried out with 100-fold dilutions of total DNA extracts from plants infected with Las strain B232.1 and Lam strain 967 (data not shown). The real-time PCR amplifications were performed using a SmartCycler II (Cepheid, Sunnyvale, CA) in a 25 μl reaction volume consisting of the following reagents at the optimized concentrations: 250 nM (each) target primer (HLBas and/or HLBaf and/or HLBam and HLBr), 150 nM target probe (HLBp), 300 nM (each) internal control primers (COXf and COXr), 150 nM internal control probe (COXp), 6.0 mM $MgCl_2$, 250 μM dNTPS, 1 × PCR buffer and 1 unit Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The standard amplification protocol was 95 °C for 20 s followed by 40 or 50 cycles at 95 °C for 1 s and 58 °C for 40 s. All reactions were performed in triplicate and each run contained one negative and one positive control. The data were analyzed using the Smart Cycler® software Version 2.0D.

3. Results

3.1. Specificity of real-time TaqMan PCR assays for Ca. Liberibacter spp.

The specificity of the assays with the primer–probe combinations, HLBas-HLBp-HLBr (HLBaspr), HLBam-HLBp-HLBr (HLBampr) and HLBas-HLBam-HLBp-HLBr (HLBasampr) was evaluated in single and multiplex real-time PCR with the positive internal control primer–probe combination COXf-COXp-COXr (COXfpr), using total DNA extracts from sweet orange plants infected with six strains of Las, six strains of Lam, one DNA mixture of Las strain 892 and Lam strain 974, CTV, CSD and CB, and with genomic DNA from pure cultures of other citrus bacterial pathogens or endophytes (Table 1).

The assays with HLBaspr yielded positive results (Ct values > 0) only from DNA samples infected with Las and negative results (Ct values = 0) from samples

Table 1
Specificity of quantitative real-time TaqMan PCR assays for *Candidatus Liberibacter* species in plant extracts

Species, strain or isolate	Host	Provider	Origin	Ct values with primers[a] specific to		
				Las	Lam	Las/Lam
Candidatus Liberibacter asiaticus B239	*Citrus* sp.	Hartung, J.S.	Taiwan	22.51	0	22.26
Candidatus Liberibacter asiaticus KIN1	*Citrus* sp.	Iwanami, T.	Japan	25.26	0	24.89
Candidatus Liberibacter asiaticus IDN5	*Citrus* sp.	Iwanami, T.	Indonesia	26.32	0	25.99
Candidatus Liberibacter asiaticus 892	*Citrus* sp.	Teixeira, D.C.	Brazil	21.61	0	21.05
Candidatus Liberibacter asiaticus 919	*Citrus* sp.	Teixeira, D.C.	Brazil	24.29	0	24.25
Candidatus Liberibacter asiaticus 969	*Citrus* sp.	Teixeira, D.C.	Brazil	20.93	0	20.12
Candidatus Liberibacter americanus 974	*Citrus* sp.	Teixeira, D.C.	Brazil	0	23.67	23.24
Candidatus Liberibacter americanus 975	*Citrus* sp.	Teixeira, D.C.	Brazil	0	22.65	22.42
Candidatus Liberibacter americanus 976	*Citrus* sp.	Teixeira, D.C.	Brazil	0	24.11	23.98
Candidatus Liberibacter americanus 077	*Citrus* sp.	Teixeira, D.C.	Brazil	0	24.43	24.15
Candidatus Liberibacter americanus 978	*Citrus* sp.	Teixeira, D.C.	Brazil	0	23.81	23.62
Candidatus Liberibacter americanus 979	*Citrus* sp.	Teixeira, D.C.	Brazil	0	23.97	23.58
DNA mixture of 892 and 974	*Citrus* sp.	Teixeira, D.C.	Brazil	22.21	23.56	21.88
Xanthomonas axonopodis pv. *citri* strain A	*Citrus* sp.	Mavrodieva, V.	USA	0	0	0
Xylella fastidiosa CVC strain L001	*Citrus* sp.	Hartung, J.S	Brazil	0	0	0
Phytophthora citricola I 22F3	*Citrus* sp.	Mavrodieva, V.	Unknown	0	0	0
Phytophthora citrophthora I 1E4	*Citrus* sp.	Mavrodieva, V.	Unknown	0	0	0
Methylobacterium mesophilicum (SR1.6/6)	*Citrus* sp.	Hartung, J.S.	Brazil	0	0	0
Curtobacterium flaccumfaciens (ER1/6)	*Citrus* sp.	Hartung, J.S.	Brazil	0	0	0
Pantoeo agglomerans (ARB18)	*Citrus* sp.	Hartung, J.S.	Brazil	0	0	0
Enterobacter cloacae (PR2/7)	*Citrus* sp.	Hartung, J.S.	Brazil	0	0	0
Bacillus sp. (CL16)	*Citrus* sp.	Hartung, J.S.	Brazil	0	0	0
Citrus tristeza virus T36/total plant DNA	*Citrus* sp.	Hartung, J.S.	USA	0	0	0
Citrus blight/total plant DNA	*Citrus* sp.	Hartung, J.S.	USA	0	0	0
Citrus sudden death virus/total plant DNA	*Citrus* sp.	Hartung, J.S.	Brazil	0	0	0

[a] HLBaspr is specific to *Ca. L. asiaticus* (Las); HLBampr is specific to *Ca. L. americanus* (Lam); HLBasampr is specific to both Las and Lam.

infected with Lam or any other citrus pathogens or endophytes tested. The assays with HLBampr produced positive results only from samples infected with Lam. The assays with multiple primer–probe combination HLBasampr detected HLB pathogens in samples infected with Las and/or Lam and yielded negative results for all other citrus pathogens and endophytes tested (Table 1). The results demonstrated that primer–probe combinations HLBaspr and HLBampr were specific to Las and Lam, respectively, and do not have cross-reactivity with other pathogens or endophytes commonly resident in citrus. The specificity of the two primer–probe combinations was retained in multiplex PCR assays with the multiple primers.

3.2. Sensitivity of real-time TaqMan PCR assays for Ca. Liberobacter spp.

Since HLB pathogens (*Candidatus Liberibacter* sp.) are to date nonculturable, serial dilutions of total DNA extracts obtained from HLB symptomatic greenhouse-grown plants infected with Las strain B239 (Table 1) and from plants infected with Lam strain 988 in the field in Brazil were used to evaluate the sensitivity of primer–probe combinations in single or multiplex PCR. The primer–probe set HLBaspr, specific for Las, detected Las in 1 μl of the 10^{-5} dilution of the DNA sample B239 (Fig. 2, Table 2). This DNA was extracted using a DNeasy Plant Kit (Qiagen, Valencia, CA) from 200 mg of midribs of symptomatic leaves of a greenhouse-grown sweet orange tree. The original total DNA was suspended in 100 μl of TE buffer. One microliter of the 10^{-5} dilution from the original DNA sample contained fresh weight (FW) equivalent of 20 ng of the midribs from HLB symptomatic leaves (200 mg ÷ 100 μl × 10^{-5} × 1 μl). This was the sensitivity of HLBaspr when total DNA was extracted from midribs of greenhouse-grown plant material using Qiagen kits. A linear standard curve was generated from the serial dilutions of this total plant DNA sample (Fig. 3).

The primer–probe set HLBampr specific to Lam detected Lam in 1 μl of the 10^{-4} dilution of the DNA sample 988 (Fig. 4, Table 2). The DNA sample was obtained by CTAB extraction of 500 mg of midribs of symptomatic leaves collected from a HLB-symptomatic, field-grown, sweet orange tree in, São Paulo, Brazil. The total DNA was originally suspended in 100 μl of water. One microliter of the 10^{-4} dilution from this original DNA sample contained FW equivalent of 500 ng of midribs of HLB symptomatic leaves (500 mg ÷ 100 μl × 10^{-5} × 1 μl). This was the sensitivity of HLBampr when total DNA was extracted from petioles of field-grown plant materials using the CTAB method.

The positive internal control primer–probe set COXfpr yielded a positive result for COX genes of host plants in 1 μl of the 10^{-7} dilution of the DNA sample 988 (Fig. 5, Table 2). The extract was of quality sufficient to detect plant DNA in an aliquot equivalent of 0.5 ng of plant midribs (500 mg ÷ 100 μl × 10^{-7} × 1 μl). However, when total DNA of a plant infected with Las strain B239 was extracted using a Qiagen kit, the greatest dilution at which host plant DNA could be detected was 10^{-5} (1 μl) (Table 2). This portion of the DNA extract was from about 20 ng of plant materials.

The sensitivities of the three primer–probe sets in single PCR reactions were retained in multiplex PCR assays (Table 2). Multiple channels in the optical system of the real-time PCR machine were successfully used to collect the data from the reactions with the primer–probe set(s) specific to the target DNA of *Ca.*

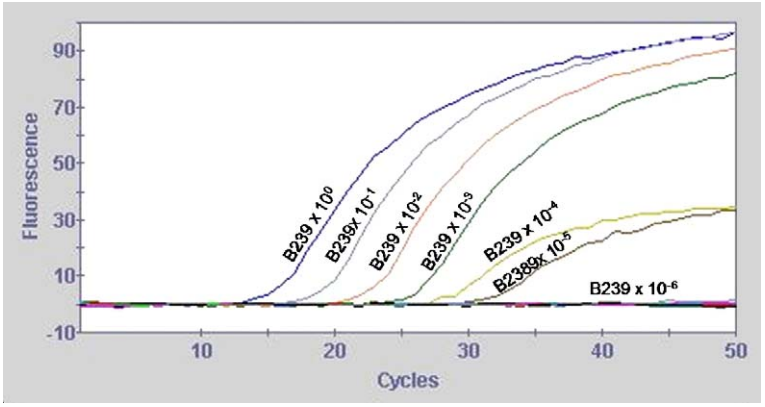

Fig. 2. Sensitivity of the primer–probe combination HLBaspr specific to *Ca. L. asiaticus* in multiplex real-time PCR with the positive internal control primer–probe set COXfpr. Templates were serial dilutions of a total DNA sample extracted from a greenhouse-grown, HLB symptomatic, sweet orange plant inoculated with *Ca. L. asiaticus* strain B239.

Table 2
Sensitivity of quantitative real-time TaqMan PCR assays for *Candidatus Liberibacter* species in plant extracts

DNA serial dilution	Cycle threshold (Ct) values of quantitative real-time PCR													
	Singleplex PCR[a]				Multiplex PCR of target(s) and host DNA[b]									
	Of target DNA		Of host DNA		*Ca. L. asiaticus* strain B239						*Ca. L. americanus* strain 988			
	B239	988	B239	988	HLBaspr/ COXfpr		HLBasampr/ COXfpr		HLBasafpr/ COXfpr		HLBampr/ COXfpr		HLBamaspr/ COXfpr	
	FAM	FAM	TET	TET	FAM	TET	FAM	TET	FAM	TET	FAM	TET	FAM	TET
10^{0}	17.22	24.25	14.99	16.83	17.22	15.05	17.36	15.27	17.11	15.13	24.25	16.83	24.18	16.66
10^{-1}	20.31	27.41	18.40	19.83	20.14	18.55	20.20	18.64	20.02	18.45	27.41	19.83	27.24	19.70
10^{-2}	23.83	30.83	21.48	23.75	23.59	21.63	23.66	21.86	23.15	21.53	30.83	23.75	30.66	23.56
10^{-3}	27.29	33.54	25.80	26.81	27.02	25.24	27.18	25.48	27.00	25.21	33.54	26.81	33.37	26.68
10^{-4}	31.24	36.20	29.13	30.26	31.01	29.02	31.26	29.33	31.12	29.09	36.20	30.26	36.05	30.08
10^{-5}	33.99	0	32.14	33.37	33.68	32.22	33.82	32.77	33.56	32.32	0	33.37	0	33.19
10^{-6}	0	0	0	37.39	0	0	0	0	0	0	0	37.39	0	37.22
10^{-7}	0	0	0	39.16	0	0	0	0	0	0	0	39.16	0	39.04
10^{-8}	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Water	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] In singleplex PCR reactions, only one prime–probe set (HLBaspr for Las B239 or HLBampr for Lam 988) or one internal control primer–probe set COXfpr for host DNA was used.
[b] In multiplex PCR reactions, multiple primer–probe sets were used; HLBaspr is specific to *Ca. L. asiaticus* (Las); HLBampr is specific to *Ca. L. americanus* (Lam); HLBasampr is specific to both Las and Lam; HLBasafpr is specific to both Las and *Ca. L. africanus* (Las); HLBamaspr is specific to both Lam and Las.

Liberibacter spp. and from those with the positive internal control primer–probe set (Fig. 6). Two species of the *Ca. Liberibacter* were simultaneously detected in a single reaction tube (Fig. 7, Table 2). All reactions with any of the primer–probe combinations in single or multiplex PCR were negative for the no template (water only) controls (Table 2).

3.3. TaqMan PCR assays of HLB-infected plant samples

DNA samples from 77 plants (76 from sweet orange and 1 from periwinkle) inoculated with Las strains by budding were analyzed by multiplex quantitative real-time PCR with HLBaspr and COXfpr or with HLBampr and COXfpr. Seventeen of the 76 DNA samples from sweet orange plants were positive for Las with Ct values between 20 and 30. These results were consistent with those of HLB symptom observation and conventional PCR with the primer sets OI2c/OI1 (Jagoueix et al., 1996), A2/J5 (Hocquellet et al., 1999), Hung1 and Hung2 (Hung et al., 1999), and Las1/Las2 (Li, 2005, unpublished) (data not shown), with the exception of one DNA sample which was positive for Las by real-time PCR but negative by conventional PCR assays or symptom observation. The DNA sample from a periwinkle plant inoculated with Las by dodder (*Cuscuta campestris*) was also positive for Las by real-time PCR

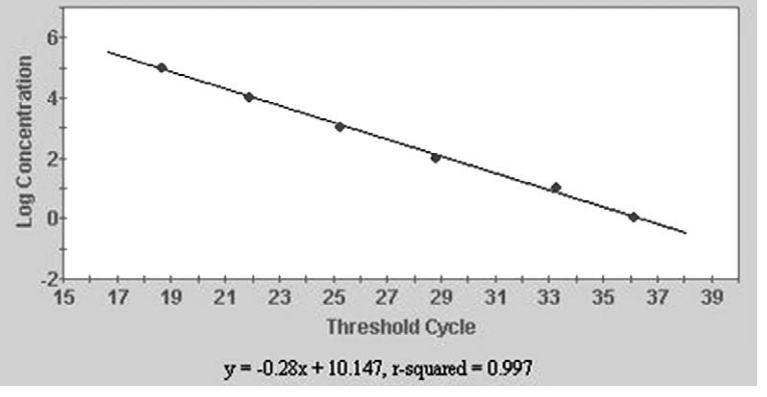

Fig. 3. A standard curve obtained in multiplex real-time PCR with primer–probe combinations HLBaspr and COXfpr using the serial dilutions (10^{0}–10^{-6}) of a DNA extract of a greenhouse-grown, HLB symptomatic, sweet orange plant inoculated with *Ca. L. asiaticus* strain B239.

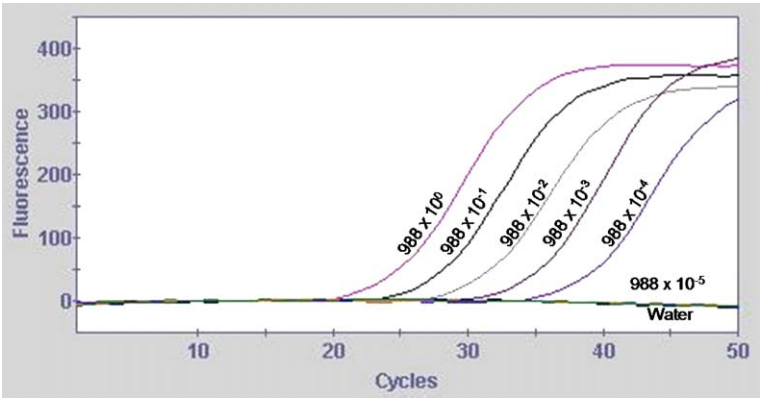

Fig. 4. Sensitivity of the primer–probe combination HLBampr specific to *Ca. L. americanus* in multiplex real-time PCR with the positive internal control primer–probe set COXfpr using the serial dilutions of a DNA sample extracted from a field-grown, HLB symptomatic sweet orange plant (#988) from Araraquara, São Paulo, Brazil.

and conventional PCR (data not shown). The two DNA samples received from Japan, KIN1 and IDN5, were also positive for Las by real-time PCR and loop-mediated isothermal amplification (LAMP) (Okuda et al., 2005). None of the DNA samples above were positive for Lam.

Among 25 DNA samples extracted from orange trees from São Paulo, Brazil, 2 were negative for HLB pathogens, 3 were positive for Las and 20 were positive for Lam. The Ct values of the positive DNA samples were between 20 and 26. No mixed infections of Las and Lam were detected by real-time PCR in these DNA samples, and no discordance was observed between the results of real-time PCR and those of conventional PCR (data not shown). All of the 24 DNA extracts prepared by us from the eight plant samples received for confirmation of HLB in Florida were positive for Las, with Ct values between 20 and 32, and negative for Lam (Table 3). The high quality and consistency of DNA extracts prepared from these samples was shown by the TET Ct values obtained for the internal (COX) control (Table 3).

Multiplex real-time PCR assays were also run with 10- and 100-fold dilutions of all 104 DNA samples above. No additional positive results occurred, indicating that citrus plant inhibitors did not affect the results of real-time PCR.

4. Discussion

Because none of the species of *Ca. Liberibacter* is culturable, only a few fragments of their genomic DNA have been cloned and sequenced (Coletta-Filho et al., 2005; Hung et al., 1999; Jagoueix et al., 1994, 1997; Subandiyah et al., 2000; Teixeira et al., 2005c; Villechanoux et al., 1993). The best characterized regions are the 16S rDNA and the 16S/23S intergenic regions (Coletta-Filho et al., 2005; Jagoueix et al., 1994, 1997;

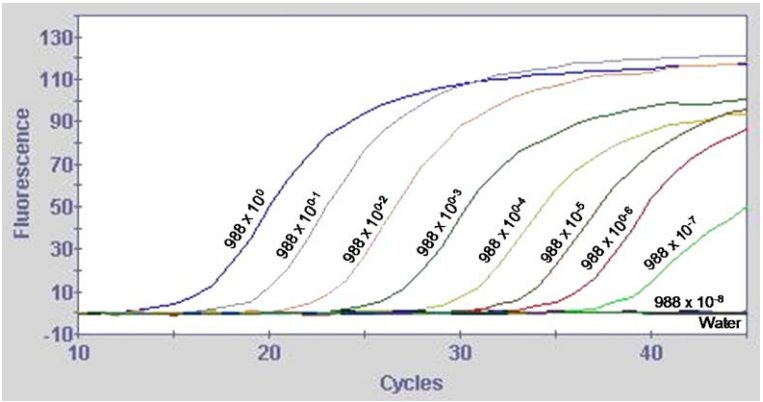

Fig. 5. Sensitivity of the positive internal control primer–probe set COXfpr in multiplex real-time PCR with the target primer–probe set HLBampr using the serial dilutions of a DNA sample obtained from a field-grown, HLB symptomatic sweet orange tree (#988) from Araraquara, São Paulo, Brazil.

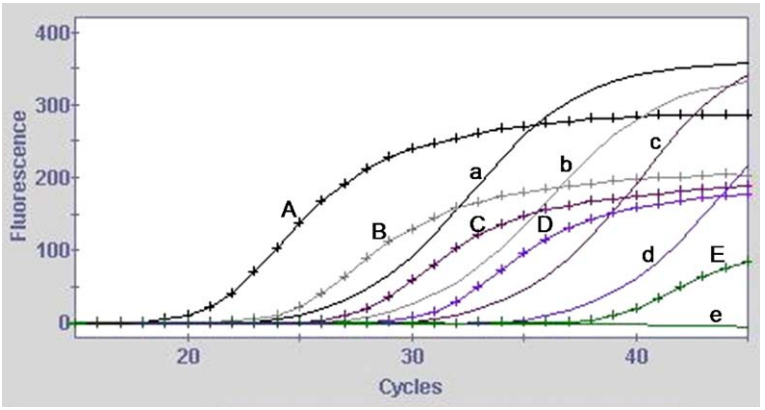

Fig. 6. A multiplex real-time PCR of serial dilutions (10^{-1}–10^{-4}) of DNA sample #988, from a field-grown, HLB symptomatic sweet orange tree from São Paulo, Brazil. Fluorescence data from channel 1 (plain lines a, b, c, d and e) for detection of the target DNA of *Ca. L. americanus* with the primer–probe set HLBampr and from channel 3 (asterisk-marked lines, A, B, C, D and E) for assessment of the quality of DNA extract with the positive internal control primer–probe set COXfpr.

Subandiyah et al., 2000; Teixeira et al., 2005c). The sequences of the 16S rDNA are highly conserved among the species of *Ca. Liberibacter* spp., but variation is sufficient to design primers capable of detection and identification of the bacterium in conventional PCR assays (Coletta-Filho et al., 2005; Jagoueix et al., 1996; Teixeira et al., 2005c). Based on the variation in the 16S rDNA sequences among *Ca. Liberibacter* spp. (Fig. 1a), three TaqMan forward primers, HLBas, HLBaf and HLBam, were designed. There are three SNPs between the priming sites of HLBas and HLBaf, six SNPs and one INDEL between the priming sites of HLBaf and HLBam, three SNPs and one INDEL between the priming sites of HLBas and HLBam. HLBaspr and HLBampr were demonstrated to be specific to Las and Lam, respectively, as designed. In these assays, they were paired with the same reverse primer, HLBr, and used the same hybridization probe HLBp. Neither of the two primer–probe sets cross reacted with any other citrus pathogens or endophytes tested.

The TaqMan assays developed with the primer–probe sets HLBaspr and HLBampr were very sensitive, with a detection limit of pathogen DNA in FW equivalent to 20 and 500 ng of midribs of HLB symptomatic leaf tissues collected in the greenhouse or field, respectively. In previous work, conventional PCR amplification with the primers OI2c/OI1, also targeting the 16S rDNA, was obtained with 2–10 μl from 100 μl of original DNA extracts when 20 mg, but not when lesser amounts of infected midribs were mixed with 1 g of healthy midribs (Jagoueix et al., 1996). The sensitivity of conventional PCR with the primer set A2/J5 targeting the β-operon genes was identical or slightly superior to that of PCR with OI2c/OI1 (Hocquellet et al., 1999). Inhibitors from citrus plant tissues affect the results of conventional PCR

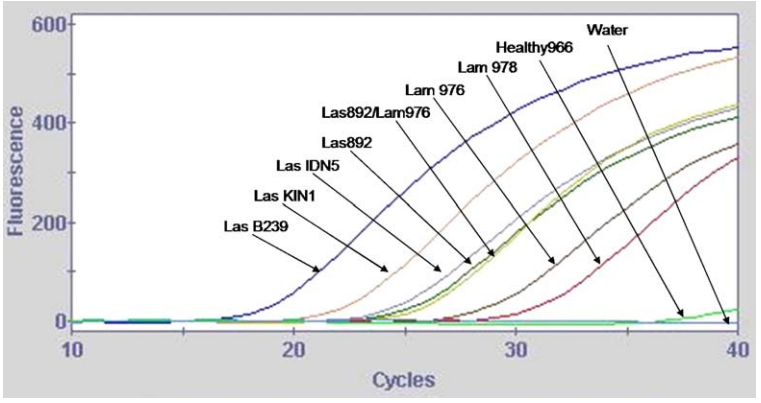

Fig. 7. Multiple species of *Ca. Liberibacter* simultaneously detected in a TaqMan PCR assay with the combined target primer–probe set HLBasampr and the positive internal control primer–probe set COXfpr.

Table 3
Multiplex real-time PCR assays for *Ca. Liberibacter* spp. in extracts of citrus plants from Florida

Source	Sample		FAM[a] Ct for pathogen DNA[a]		TET[b] Ct for plant DNA in multiplex with	
	#	Name	HLBaspr	HLBampr	HLBaspr	HLBampr
Healthy from commercial nursery source	1	Calamondin (*Citrus mitis*)	0	0	17.77	17.50
	2	Calamondin (*Citrus mitis*)	0	0	17.79	17.67
	3	Calamondin (*Citrus mitis*)	0	0	17.76	17.66
Healthy from J. Hartung	4	*Citrus sinensis*	0	0	18.47	18.45
	5	*Citrus sinensis*	0	0	17.75	17.81
	6	*Citrus sinensis*	0	0	18.72	18.50
HLB-infected plant tissues from T. Gottwald	7	Plant 7a	31.55	0	18.90	18.93
	8	Plant 7b	29.60	0	18.27	18.12
	9	Plant 7c	30.29	0	17.92	17.80
	10	Plant 4a	29.57	0	18.33	18.26
	11	Plant 4b	28.68	0	18.27	18.07
	12	Plant 4c	26.36	0	17.94	18.03
	13	Plant 2a	26.83	0	18.21	18.32
	14	Plant 2b	27.47	0	18.54	18.23
	15	Plant 2c	25.51	0	17.91	17.50
HLB-infected plant tissues from T. Schubert	16	3221a	27.56	0	17.72	17.74
	17	3221b	30.96	0	17.88	17.39
	18	3221c	29.32	0	17.51	20.97
	19	3222a	22.79	0	20.67	18.39
	20	3222b	21.15	0	18.37	18.89
	21	3222c	22.59	0	18.74	18.31
	22	3223a	24.77	0	18.40	17.52
	23	3223b	25.80	0	17.29	17.91
	24	3223c	25.48	0	18.08	19.88
HLB-infected plant tissues from T. Gottwald	25	13600a	21.72	0	19.89	18.98
	26	13600b	20.26	0	19.09	19.11
	27	13600c	20.26	0	18.97	19.12
	28	Lip1	31.58	0	20.34	20.04
	29	Lip2	31.38	0	20.16	20.04
	30	Lip3	31.90	0	20.15	19.80
Las DNA from Hartung	31	+Control	28.26	0	21.55	–
Lam DNA from Brazil		+Control	0	32.78	–	22.84
Water	32	−Control	0	0	0	0

HLBaspr is specific to *Ca. L. asiaticus*; HLBamps is specific to *Ca. L. americanus*; TET Ct values were obtained with the internal control primer–probe set COXfpr for host plat DNA.
[a] 6-Carboxy-fluorescein (FAM).
[b] Tetrachloro-6-carboxy-fluorescein (TET).

assays (Hartung et al., 1993; Jagoueix et al., 1996; Hocquellet et al., 1999; Li et al., in press). However, our TaqMan assays with dilutions of *Ca. Liberibacter*-infected total plant DNA samples demonstrated that such inhibitors did not affect the results of the real-time PCR detection when total DNA was extracted either with the standard CTAB method or with DNeasy plant kits (Qiagen, Valencia, CA). In addition, the sensitivity of our multiplex real-time TaqMan assays for detection and identification of Las and Lam was not influenced by the positive internal control reaction or by the presence in the PCR mixture of the primer specific for the other species of *Ca. Liberibacter*. The differences in sensitivity and inhibition between these TaqMan and previous conventional PCR assays are probably because the amplicon (≈70 bp) of the TaqMan assays is much smaller than that of the conventional PCR assays (≈1200 bp). Reactions targeting smaller amplicons have been shown to be less vulnerable to inhibitors of the amplification reaction (Mackay et al., 2002).

We were able to detect Las in extract equivalent to 20 ng of midribs from symptomatic leaves. Thus there is a minimum of one target sequence in 20 ng of

midribs, or a minimum of 5×10^7 Las/g of fresh midribs of original samples. By the same way, there is a minimum of 2×10^6 Lam/g of fresh midribs of original samples collected in the field in Brazil, since we were able to detect Las in extract equivalent to 500 ng of midribs. If there are multiple copies of the rRNA operon in Las or Lam genomes, as is true for example in *X. fastidiosa* (Simpson et al., 2000), the population estimates for these samples would have to be revised accordingly. The population threshold of *X. fastidiosa*, another systemic, vascular bacterial pathogen, required to induce typical symptoms in grapevines and citrus is 10^{6-8} CFU/g of fresh petioles (Hill and Purcell, 1997; Li et al., 2003). Therefore, our estimate of the bacterial populations of HLB pathogens in plants of sweet orange based on the data of quantitative PCR assays seems reasonable.

Phylogenetic analyses indicate that the Liberibacters are the closest relatives of *Bartonella henselae* and *Burucella abortus* in α division of the *Proteobacteria* (Teixeira et al., 2005c). *B. henselae* is a zoonotic agent and its genome is 1.9 Mb (Alsmark et al., 2004). The complete genome of *B. abortus*, one of the causal agents of Brucellosis, a bacterial disease of animal that can be transmitted to humans, is 3.3 Mb (Halling et al., 2005). Assuming that the genome size of *Ca. Liberibacter* species is equal to the mean of the genomes of its two closest relatives, the estimated size would be 2.6 Mb. One copy of genomic DNA would be about 4.0 fg. According to the estimates of bacterial populations in plant tissues discussed above, the DNA sample #988 of Lam should have at least 1×10^6 copies ($\approx$4.0 ng) of genomic DNA of the pathogen (Fig. 4). The total DNA concentration of sample 988 was 515μg/ml, as estimated with a spectrophotometer. So, 100 μl (final elution volume) of the DNA sample #988 should have 51.5 μg of total DNA. Therefore, the ratio of HLB pathogen DNA to plant DNA should be about 1:13,000 (w/w). The slope of fluorescence curves obtained in real-time PCR is a measure of the PCR efficiency (Pfaffle, 2001). The reactions with the primer–probe set specific to the pathogen DNA (Fig. 4) and those with the internal control primer–probe set targeting the host plant DNA (Fig. 5) had the same slope of fluorescence curves, indicating the same PCR efficiency for these two kinds of PCR reactions. Therefore, the difference in the detection limits between the two kinds of reactions could be used to estimate the ratio of HLB pathogen DNA to host plant DNA in terms of target copies (c/c), which was clearly shown to be 1:1000. The difference between the ratio in DNA mass and that in target DNA copies should be mainly due to the difference in the genome size between HLB pathogens and citrus plants.

A similar ratio of pathogen DNA to plant DNA was found in the two DNA samples from rough lemon plants infected with. Las strains KIN1 and IDN5 from greenhouse-grown citrus in Japan, as well as the three DNA samples from sweet orange plants infected by Las and the 20 Las samples from Brazil (data not shown). The estimates above shed light on why only three Las-specific clones were obtained out of 836 *Escherichia coli* colonies that had been transformed with total DNA extracts from Las infected midribs of Tankan tangor (*Citrus tankan* Hay.) (Hung et al., 1999). In addition, high concentrations of total DNA, up to 600 ng/μl, did not inhibit the TaqMan assays, confirming that CTAB extraction is among the best methods for sample preparation prior to PCR amplification for detection and identification of pathogens in citrus tissues (Li et al., in press).

Two species, Las and Lam, recently have been reported in Brazil (Coletta-Filho et al., 2004; Teixeira et al., 2005a). Among the HLB-symptomatic citrus plants in Brazil, about 98%, 1% and 1% of them were infected by Lam, Las and both *Ca*. species, respectively (Teixeira et al., 2005b). It seems that the Asian citrus psyllid, *D. citri* Kuwayama, can transmit both Las and Lam (Teixeira et al., 2005b). This psyllid has become established in Florida (Halbert and Manjunath, 2004), and HLB caused by Las was recently detected and confirmed in Florida (Knighten et al., 2005). Research is greatly needed on rapid and robust diagnosis, in order to facilitate research on HLB disease epidemiology and disease management. Quantitative TaqMan PCR technology offers many advantages over conventional PCR assays, including rapidity, efficiency, elimination of carry-over contamination and quantification (Brancart et al., 2005). Our combined TaqMan primer–probe set, HLBasampr was designed for assays of both Las and Lam. The multiplex TaqMan real-time PCR protocol with the combined primer–probe set and the positive internal control TaqMan primer–probe set COXfpr has been proven to be rapid, reliable, specific, sensitive and able to detect simultaneously both Lam and Las in complex DNA samples. Use of this multiplex TaqMan assay will be very helpful for quarantine laboratories and disease management and research programs.

We have also designed a TaqMan primer–probe set for real-time PCR detection of Laf (Fig. 1). Unfortunately, samples of Laf were not available to us for study. The sensitivity of the TaqMan assays with HLBafpr should be further evaluated using naturally infected

environmental samples from Africa, the Arabian Peninsula and the Mascarene Islands (Hocquellet et al., 1999).

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