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Extraction of DNA from Orange Juice, and Detection of Bacterium *Candidatus* Liberibacter asiaticus by Real-Time PCR

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ABSTRACT: Orange juice processed from Huanglongbing (HLB) affected fruit is often associated with bitter taste and/or off-flavor. HLB disease in Florida is associated with *Candidatus* Liberibacter asiaticus (CLas), a phloem-limited bacterium. The current standard to confirm CLas for citrus trees is to take samples from midribs of leaves, which are rich in phloem tissues, and use a quantitative real-time polymerase chain reaction (qPCR) test to detect the 16S rDNA gene of CLas. It is extremely difficult to detect CLas in orange juice because of the low CLas population, high sugar and pectin concentration, low pH, and possible existence of an inhibitor to DNA amplification. The objective of this research was to improve extraction of DNA from orange juice and detection of CLas by qPCR. Homogenization using a sonicator increased DNA yield by 86% in comparison to mortar and pestle extraction. It is difficult to separate DNA from pectin; however, DNA was successfully extracted by treating the juice with pectinase. Application of an elution column successfully removed the unidentified inhibitor to DNA amplification. This work provided a protocol to extract DNA from whole orange juice and detect CLas in HLB-affected fruit.

KEYWORDS: *Huanglongbing, greening disease, Candidatus Liberibacter asiaticus, orange juice, amplification inhibitor, 16S rDNA, qPCR*

INTRODUCTION

Huanglongbing (HLB) or citrus greening disease in Florida is widespread and associated with *Candidatus* Liberibacter asiaticus (CLas), a phloem-limited bacterium. This disease can kill a tree in 5−10 years, and orange juice processed from HLB-affected fruit is often associated with bitter taste and/or off-flavor.[1,2] CLas population has been shown to correlate with HLB symptoms, and those leaves with serious symptoms have higher CLas population.[3−5] Among numbers of diagnostic methods to detect CLas, APHIS recommended quantitative real-time PCR (qPCR) analysis targeting the CLas 16S rDNA gene using TaqMan protocol.[6] In comparison to midribs of leaves, which are rich in phloem vessels and used as a standard for HLB diagnostics for trees, fruit juice vesicles contain much lower CLas,[7,8] and this increases the difficulty for CLas detection in orange juice. Standard deviation of the cycle threshold (Ct) value in real-time PCR increases as target DNA decreases, indicating a higher risk of error at low target DNA concentrations.[9] Although many methods have succeeded in extraction of high-quality DNA from midribs of leaves for CLas detection,[4−6] our attempts to isolate DNA from orange juice using Qiagen's DNeasy Plant mini kit, Food kit, QIAmp Blood kit, or Promega's Wizard Genomic DNA purification kit were unsuccessful. Other problems obtaining pathogenic DNA from plant tissues include the physical restriction of cell walls,[10−12] and for CLas bacteria that are localized in phloem sieve cells, lysis is even more difficult.[13] Often DNA extraction is accomplished using a mortar and pestle under liquid nitrogen where the finer the grind the more DNA yield;[14] however, for microorganisms in plant tissues sometimes sonication, enzymatic digestion, or other lysis steps are required.[10,15−19] DNA isolation from plants is further complicated by the presence of pectin, which can coprecipitate with DNA[20] when alcohol is used in the extraction process. Pectinase can then be efficient, environmentally friendly, and economical.[14]

Another common challenge for extracting quality DNA is that the extract is often contaminated with secondary metabolites and/or humic acid, which can inhibit PCR reaction.[10−14] Orange juice is rich in secondary metabolites, including alkaloids, limonoids, and flavonoids.[1,15] Inhibitors from citrus plant tissues affect the results of conventional PCR assays.[16−18] Appropriate ion-exchange columns or chelating agents can be used to remove contaminants.[19] Kim and Cho[11] successfully removed PCR inhibitors from apple, grape, and watermelon juices using Chelex treatment and Sephadex column filtration. However, they did not succeed by this method for orange juice.[11] Li et al.[6] showed that TaqMan assays were not inhibited when the leaf samples were extracted with the standard cetyltrimethyl ammonium bromide (CTAB) or the DNeasy plant kit (Qiagen), indicating that TaqMan assays with a small amplicon (about 70 bp) perhaps are less vulnerable to inhibitors of amplification reaction in comparison with the conventional PCR assays with a large amplicon (about 1200 bp).[20]

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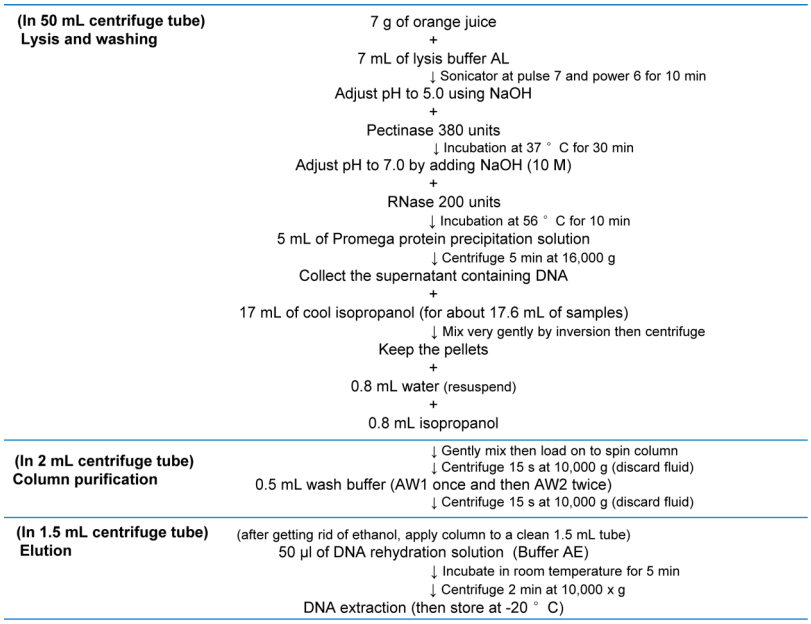

Figure 1. Standard DNA extraction protocol from orange juice for preparation of qPCR samples.

A number of protocols have been established for extraction of DNA from orange juice without the need for enrichment or isolation of microbial targets.[21] However, the procedures included precipitation of bacteria or spores prior to extraction of DNA.[21] Because of the low ratio of live cells (17−31% in citrus samples)[4,22] and because dead CLas cells have already caused metabolic changes in plants and fruit,[8,23] a new method to extract CLas DNA from whole juice, including DNA in dead and broken cells, is considered necessary.

The primary objective of this study was to develop a streamlined protocol to efficiently extract genomic DNA from processed orange juice made from fruit harvested from HLB-diseased trees and detect CLas population in the juice. This paper will also discuss the importance of using a relative Ct value by comparing the reference plant cytochrome oxidase DNA (COX) Ct with the target (16S rDNA) Ct. Detection of CLas DNA in processed orange juice will provide useful information for processors to determine if juice is coming from HLB-infected fruit.

MATERIALS AND METHODS

Plant Materials and Juice Processing. Two major Florida juice oranges (*Citrus sinensis* (L.) Osbeck) were used: early maturing 'Hamlin' and late-maturing 'Valencia'. HLB symptomatic (HLBs), asymptomatic (HLBa), and healthy fruit for each cultivar were harvested at commercial maturity from a commercial grove located in south Florida in the 2010 and 2011 seasons. Both HLBs and HLBa fruit were harvested from HLB-infected trees, which were visually symptomatic for the disease,[24] and real-time PCR was used to detect CLas in leaf midribs[6] to confirm infection. HLBs fruit were misshapen, small, and green, while HLBa fruit were similar to healthy fruit in size, color, and shape and usually were located on the asymptomatic sectors of HLB-infected trees.

Fruit were processed into juice directly after harvest by standard industry procedure. Briefly, fruit were fed into an industrial cup extractor (JBT 391, JBT Food Tech, Lakeland, FL); pulp was reduced using a pressure filtration finisher with screen size of 0.51 mm (JBT) and then thermopasteurized using a pilot pasteurizer (UHT/HTST Lab 25EHV Hybrid, Microthermics, Inc., Raleigh, NC) at 90 °C for 10 s with a flow rate of 1.2 L min^{-1}. Each treatment contained at least 100 kg of fruit, and four replicate juice samples were taken upon exiting the pasteurizor at regular intervals. Juice samples were stored at −20 °C until used for DNA extraction.

HLBs and healthy juice samples processed from 'Valencia' oranges harvested on May 10, 2010 were used for development of CLas DNA detection methodology. All other juices including 'Valencia' harvested on April 21, 2011 and Hamlin harvested on February 3, 2010 and December 15, 2010 were used to confirm the new protocol.

Leaf samples from the same 'Valencia' trees were used to compare to the juice samples in an experiment to compare methodologies.

Chemicals and Reagents. Most of the DNA extraction kits, spin columns, and buffers were from Qiagen (Valencia, CA) including the DNeasy mericon Food kit, DNeasy Plant Maxi kit, QIAamp DNA Blood Mini kit, Buffer AE, Buffer AL, Buffer AW1, Buffer AW2, QIAamp Mini Spin column, DNA. The Wizard Genomic DNA purification kit, pGME T-easy vector, and Protein precipitate solution were from Promega (Madison, WI). Pectinase produced by *Aspergillus niger*, 200 proof absolute ethanol, Molecular Biology-grade 2-propanol, and sterile water were from Sigma-Aldrich (St. Louis, MO). A TaqMan Universal Master Mix II and all primer and probe DNA sequences were manufactured by Applied Biosystems (Foster City, CA).

Genomic DNA Extraction from Juice. Figure 1 shows the newly developed standard extraction protocol for DNA extraction from orange juice. Components of the Wizard Genomic DNA purification kit and QIAamp DNA Blood Mini kit were used in multiple steps of the protocol. Briefly, 7 g of juice was mixed with 7 mL of Buffer AL and disrupted using a sonicator (Omni Sonic Ruptor 250, Omni International, Kennesaw, GA) at a pulse 70 and power 6.5 for 10 min in an ice bath. The mixture was adjusted to pH 5.0 by adding about 25 µL of a 10 M NaOH solution prior to adding 380 units of pectinase and incubating at 37 °C for 30 min to hydrolyze pectin molecules. Following pectinase treatment, more NaOH solution (10 M, about 50 µL) was added to neutralize the mixture to pH 7.0, and 200 units of DNA-free RNase was added prior to incubation at 56 °C to hydrolyze RNA and improve DNA extraction. Then 5 mL of Promega protein precipitate solution was added to the mixture, protein precipitate was separated by centrifugation, and pellets were discarded. The supernatant containing DNA was mixed with 17 mL of cold isopropanol to precipitate DNA. After centrifugation, the DNA mixture in the pellets was dissolved in 0.8 mL of water, and then 0.8 mL of isopropanol was added prior to loading the mixture onto a QIAmp Mini Spin column. AW 1 and AW 2 buffers, 0.5 mL each, were used to wash the column. Finally, DNA was eluted in 50 µL of Buffer AE.

Table 1. Effect of Disruption and pH Adjustment on Efficiency of DNA Extraction[a]

	DNA content (ng uL^{-1})	Ct: COX	Ct: 16S rDNA
standard (pH 7.0)	565 ± 14.2 a	16.7 ± 0.1 a	29.4 ± 0.1 a
without ultrasonic disruption (pH 7.0)	286 ± 49.2 b	17.4 ± 0.2 b	30.8 ± 0.4 b
without pH adjustment (pH 4.2−4.6)	12.3 ± 1.1 c	31.6 ± 0.9 c	>40[b]
without ultrasonic disruption nor pH adjustment (pH 4.2−4.6)	10.7 ± 0.8 c	32.8 ± 1.4 c	>40

[a]DNA was extracted following the standard procedure shown in Figure 1 except as described otherwise. Values followed by the same letter within columns are not significantly different by the Tukey's test at the 0.05 level. [b]Ct > 40: not detectable.

The following experiments were conducted individually and/or in combinations to examine the key steps in the protocol.

Homogenization methods. For the ultrasonic homogenizer method, the mixture of juice sample (7.0 g) and 7.0 mL of lysis Buffer AL in a beaker submerged in an ice bath was disrupted using a 9.5 mm diameter solid tip with the ultrasonic homogenizer (Omni International) at pulse 70, power 6.5, and running time 10 min. For the traditional mortar and pestle method, a frozen juice sample was ground to a fine powder under liquid nitrogen. Then 7.0 g of frozen juice powder was mixed with 7.0 mL of Buffer AL.

Pectinase method. For pectinase treatment, pectinase (380 units) was added to a mixture of 7.0 g of juice + 7.0 mL of lysis buffer; then a NaOH solution was added to adjust the pH to 5.0, with incubation at 37 °C for 30 min. For the non-pectinase method, this step was omitted.

Neutralization of juice. For the neutralization method, NaOH solution was added to the juice with the lysis buffer extraction mixture until the pH reached 7.0 prior to adding the protein precipitate solution. On the other hand, the non-neutralization method omitted the pH adjustment step.

Use of an elution column. For the elution column method, the crude extraction was loaded onto an elution column and washed with AW 1 and AW 2 buffers, and finally, DNA was eluted with Buffer AE. For the noncolumn method, instead of AW 1 and AW 2 buffers, ethanol was used to wash off the impurities. Briefly, the pellets gained from isopropanol precipitation and centrifugation were resuspended in 70% ethanol and then centrifuged at 14 500*g* for 6 min. Pellets were subsequently washed twice using 70% ethanol by repeating the above procedure. Finally, the pellets, after air drying for 30 min, were dissolved in Buffer AE.

Individual commercial kits. Four commercial DNA purification kits were used independently following the manufacturers' instructions to compare with the standard protocol shown in Figure 1. They are the Wizard Genomic DNA Purification kit, DNeasy mericon Food kit, DNeasy Plant Maxi kit, and QIAamp DNA Blood Mini kit. Promega's Wizard Genomic DNA was selected first because it was used in our general plant tissue work, and extraction of DNA from leaf samples in this research was also conducted using this kit. Other kits were selected just because they partially match some features in orange juice being a plant product, a food, and an aqueous liquid.

The quantity of DNA samples was estimated by measuring 260 nm with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE), and purity was evaluated by examining ratios of 260/280 nm and 260/230 nm.

Quantitative PCR (qPCR). The primer/probe sequences for *C*Las 16S rDNA and citrus reference, cytochrome oxidase (COX) gene, were designed according to Li et al. (2006)[6] and manufactured by Applied Biosystems. The 16S rDNA forward primer used was 5′-TCGAGCGCGTATGCAATACG-3′, and the reverse primer used was 5′-GCGTTATCCCGTAGAAAAAGGTAG-3′. The probe used was 5′-6 FAM/AGACGGGTGAGTAACGCG/3′ TAMRA. For COX, the forward and reverse primers were 5′-GTATGCCACGTCGCATTC-CAGA-3′ and 5′-GCCAAAACTGCTAAGGGCATTC-3′, respectively. The probe was 5′-VIC/ATCCAGATGCTTACGCTGGA/3′ TAMRA. Assays of qPCR were performed using the ABI PRISM 7500 Sequence Detection Fast System (Applied Biosystems). Reactions of qPCR were performed in a 20 μL reaction using 10 μL TaqMan Universal Master Mix II, 0.25 μM each of 16S rDNA primer, 0.3 μM of each COX primer, 0.15 μM of each probe, and 2.5 μL of template DNA. PCR conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of each 95 °C for 9 s, and 60 °C for 1 min. Each plate, regardless of sample number, contained at least two negative control wells and one positive control 16S rDNA well, and each sample contained at least three replicates. Results were analyzed using ABI PRISM software. Raw data were analyzed using the default settings (threshold = 0.2) of the software. Real-time PCR for COX and 16S rDNA were run separately (simplex) and simultaneously (complex), and the results were compared.

Standard Equation for Quantification and Serial Dilution for Amplification Efficiency. The plasmid pLBA, which harbors the *C*Las 16S rDNA, was kindly provided by Dr. Niang Wang's (University of Florida) laboratory which was constructed by cloning a 1409-bp DNA fragment that was amplified using a universal primer rpl[25] and *C*Las 16S rDNA primer[26] into pGME T-easy vector. A standard equation was developed based on methods of Li et al.[7] The plasmid DNA was diluted to a 10^0−10^9 cells series per microliter in water, and 1 μL of each dilution was used for each qPCR assay. For DNA extraction from orange juice, the interval for the serial dilutions was 2^1 or 2^2 depending on the concentration of the target DNA and because of the low concentration of the target DNA.

Statistical Analysis. SAS Version 9.1 (SAS Institute, Cary, NC) was used for analysis of data, using analysis of variance (PROC ANOVA). Treatment means were separated at the 0.05 significance levels by Tukey's test. For linear regression, PROC REG was used.

RESULTS AND DISCUSSION

Effect of Homogenization Method on Isolation of DNA from the Plant Tissue. In comparison with tissue homogenization using a mortar and pestle, disruption with the sonicator increased DNA extraction about 2-fold and reduced Ct values for COX and 16S rDNA by 0.7, and 1.4, respectively, when the pH value was neutralized during extraction of DNA (Table 1). Without adjustment of pH, the effect of sonication was not evident because most of the DNA strands were lost during column filtration applied in the downstream in DNA extraction (Table 1). The significantly higher extraction efficiency in 16S rDNA (1.4 reduction of Ct) than for COX (0.7 reduction of Ct) (Table 1) indicates that *C*Las DNA was bound more tightly to the plant tissue, perhaps because the bacteria are phloem delimited.[27−29]

Pectin Gelation and Effect of Pectinase Application on Isolation of DNA. Both pectin and DNA are soluble in aqueous solutions and precipitate in alcohol.[30] When adding isopropanol and/or ethanol to precipitate DNA strands, pectin also coprecipitates. Pectin content in orange juice ranges from 0.037 to 1.433 mg g^{-1}, depending on cultivar and harvest time.[1] On the other hand, DNA content in orange juice is estimated about to be 0.3−0.5 μg g^{-1}, calculated based on the following estimation: nucleic DNA 0.68−0.98 pg per citrus cell;[31,32] <600 juice vesicles per segment;[33,34] 8 segments per orange fruit to

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produce 100 g of juice. Without removal of pectin, the DNA could not be extracted because presumably most of the DNA was trapped in the pectin gel mixture. Phenol and ethylene glycol monoethyl ether,[35,36] modified cetyl trimethyl ammonium bromide (CTAB),[37] and a high concentration of NaCl[38] have been used to extract DNA from plant tissue without coprecipitation of pectin. However, these methods either require use of hazardous organic solvents or require large quantities of agents. Our preliminary experiment also showed that the nonenzymatic methods were not powerful enough to remove such a large amount of pectin. Pectinase method is efficient, environmentally friendly, and economical to remove pectin,[39] and our data show that pectinase removed pectin in orange juice efficiently with a small amount of enzyme (15 μL per milliliter of juice sample).

Effect of Elution Columns on Removal of DNA Amplification Inhibitor. Without passing through the ion-exchange column, DNA was contaminated with orange juice components, some of them having a maximum absorbance at 230 and 280 nm (Figure 2). Serial dilution of DNA showed

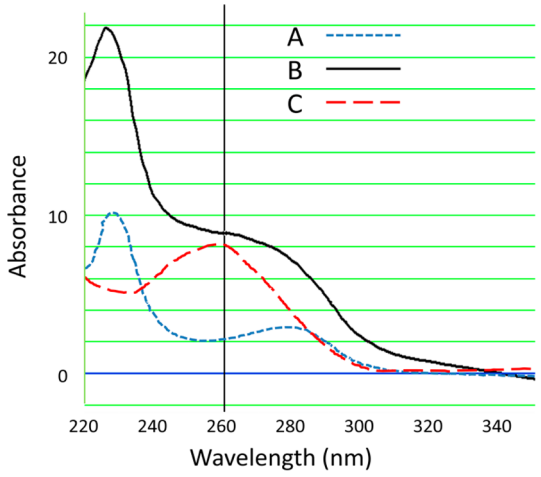

Figure 2. Absorption maximum of the extraction shifts depending upon extraction methods from 'Valencia' orange juice. Absorption spectra were recorded from 220 to 350 nm using a NanoDop 1000 Spectrophotometer: (A) DNeasy mericon Food kit, DNeasy Plant Maxi kit, and QIAamp DNA Blood Mini kit; (B) Wizard Genomic DNA purification kit; (C) standard protocol (See Figure 1).

that increase of Ct value inversely correlated with the dilution rate when extracted DNA was passed through the column (Table 2). However, without passing through the column, the increase in Ct value was not proportionate to the dilution rate (Table 2). Presumably, when 16S rDNA (the target DNA) concentration was low, it was expected to see an increase in the Ct value, but dilution also caused a decrease in the concentration of the amplification inhibitor, which, in turn, offset the effect of DNA decrease. The inhibitors in plant materials are often humic acid and other secondary metabolites.[10−14] Since humic acid is usually found in soil, plant roots, and other soil contacting organs, but not in fruit, especially juice, secondary metabolites are likely to be the major contaminant inhibitors.[11,21,40−42] Kim and Cho[11] showed that the contaminants in orange juice are more difficult to remove by column filtration than juices derived from other fruits, such as apples, grapes, and watermelons. Nevertheless, we successfully removed the inhibitors as confirmed by the qPCR results obtained in the serial dilutions of DNA extraction.

Possibly, the sensitivity of qPCR was influenced not only by PCR inhibitors discussed previously but also by nontarget DNA.[7,43,44] Nevertheless, this study showed that the nontarget DNA did not inhibit amplification of either 16S rDNA or COX when other inhibitors were not present (Table 2). However, even the amplification of 16S rDNA was remarkably influenced by inhibitors, whereas COX, with $2^{12.4}$−$2^{12.7}$ times higher DNA template concentration than 16S rDNA, was not influenced by inhibitors, even when multiplex PCR was employed (Table 2).

Within the same dilutions, the standard deviations among replicates (n = 3) also showed high variation when DNA extraction did not pass through the column (Table 2), indicating the complicated interference in DNA amplification between concentrations of templates and inhibitors.

Effect of pH on DNA Extraction. Orange juice is acidic with a pH range of 3.5−3.8.[1] After homogenizing with lysis buffer, the pH values were about 4.2−4.6. Without adjusting pH, the extraction after passing through the column showed absorbance spectrum peaks at 230 and 280 nm. However, there was no sound peak at 260 nm typical of DNA (Figure 2). Calculated DNA content, based on the reading of absorbance at 260 nm, was 535−615 ng μL^{-1}, and 16S rDNA were not able to be detected by qPCR (Table 1). However, in the standard protocol, adjusting pH to 7.0 and passing the extraction through the column, the extract showed a typical DNA absorbance spectrum with the absorption peak at 260 nm, A_{260}/

Table 2. Effect of Spin Column Application and Multiplex on PCR Amplification of 16S rDNA and COX[a]

	Ct		ΔCt	Ct		ΔCt
dilution factor	16S rDNA	COX	(Ct $_{16S\ rDNA}$ − Ct $_{COX}$)	16S rDNA	COX	(Ct $_{16S\ rDNA}$ − Ct $_{COX}$)
	noncolumn and simplex			with column and simplex		
$\times 2^0$	35.2 ± 2.3	16.3 ± 0.2	18.9 ± 2.1	29.2 ± 0.1	16.7 ± 0.1	12.5 ± 0.2
$\times 2^2$	31.1 ± 1.1	18.4 ± 0.1	12.7 ± 1.2	31.4 ± 0.2	18.7 ± 0.1	12.7 ± 0.1
$\times 2^4$	32.9 ± 0.4	20.5 ± 0.1	12.4 ± 0.4	33.4 ± 0.1	20.8 ± 0.2	12.6 ± 0.2
$\times 2^6$	35.0 ± 0.3	22.4 ± 0.1	12.6 ± 0.4	35.2 ± 0.3	22.8 ± 0.1	12.4 ± 0.3
	noncolumn and multiplex			with column and multiplex		
$\times 2^0$	>40[b]	16.5 ± 0.2		36.0 ± 2.1	16.8 ± 0.1	19.2 ± 2.0
$\times 2^2$	39.3 ± 0.6[c]	18.6 ± 0.3	20.7	39.4 ± 0.5	19.0 ± 0.2	20.4 ± 0.4
$\times 2^4$	>40	20.7 ± 0.1		>40	20.9 ± 0.1	
$\times 2^6$	>40	22.6 ± 0.2		>40	22.8 ± 0.2	

[a]DNA was extracted following the standard procedure showed in Figure 1 except as described otherwise. Serial dilution by water was used. [b]Ct > 40: not detectable. [c]Average of three replicates. Ct value of the fourth replicate was not detectable.

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A_{280} ratio of 1.94, and A_{260}/A_{230} ratio of 1.62. The effect of pH on DNA extraction can be caused by degradation of DNA or contaminants or by loss of DNA due to the decreased binding force to the silicon column.[45,46]

Effect of Multiplex Real-Time PCR on COX and 16S rDNA Amplification. When multiplex real-time PCR was applied for extraction of COX and 16S rDNA, amplification of 16S rDNA was significantly influenced by COX as shown by an irregular amplification curve (not a regular steep curve), increased Ct value, and disproportional serial dilution vs ΔCt ($Ct_{16S\ rDNA} - Ct_{COX}$) change (Figure 3 and Table 2). On the other hand, amplification of COX was not influenced by 16S rDNA (Figure 3 and Table 2). Results obtained from simplex real-time PCR for COX and 16S rDNA showed that the copy number of COX was 2^{14}–$2^{24.6}$ times more abundant than that of 16S rDNA (Table 2). It is likely because of this reason COX, which is more abundant due to greater initial quantity, performed better than for16S rDNA from the beginning, using up the deoxynucleoside triphosphates (dNTPs) in the reaction and leaving little for the other assay.[47] This problem should be overcome by limiting the amount of primer for the more abundantly expressed target (COX).[47]

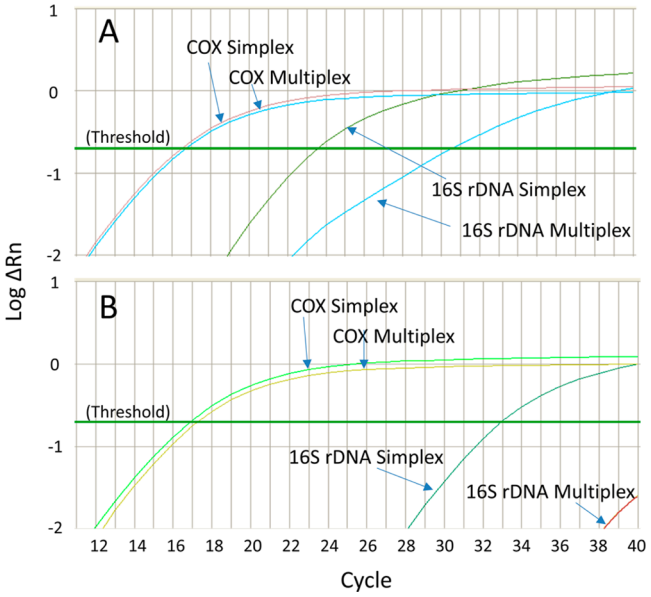

Figure 3. Amplification plots of citrus COX and Las 16S rDNA run by simplex and multiplex real-time PCR: (A) HLB-infected leaf sample; (B) HLB-infected juice sample. Note: inhibition of amplification of 16S rDNA by multiplex run was shown by increased cycle number and shape change of the amplification curve.

Multiplex-PCR is a variant of PCR which enables amplification of multiple targets in one reaction using more than one pair of primers/probes. Multiplex assays can be tedious and time consuming since they require lengthy optimization procedures, although once optimized it is a cost-saving technique used in many diagnostic laboratories.[48] The technique is subject, however, to certain difficulties related in principal to the availability of primers for various plant pathogens[49,50] and formation of primer dimers.[51] Li et al.[6] successfully developed a multiplex system to detect CLas and the citrus reference (COX). However, our preliminary experiment showed that 16S rDNA amplification was remarkably inhibited by the multiplex method. Thus, optimization of reaction conditions should aim to minimize such nonspecific reactions and avoid false results (Figure 3).[52,53]

Copy Numbers of 16S rDNA and CLas Population in Orange Juice. A standard curve was developed using a serial dilution of plasmid 16S rDNA containing 10^0–10^9 copies (Figure 4A) along with a regression analysis (Figure 4B). Copy numbers of 16S rDNA in each reaction (well) can be calculated by the following equation

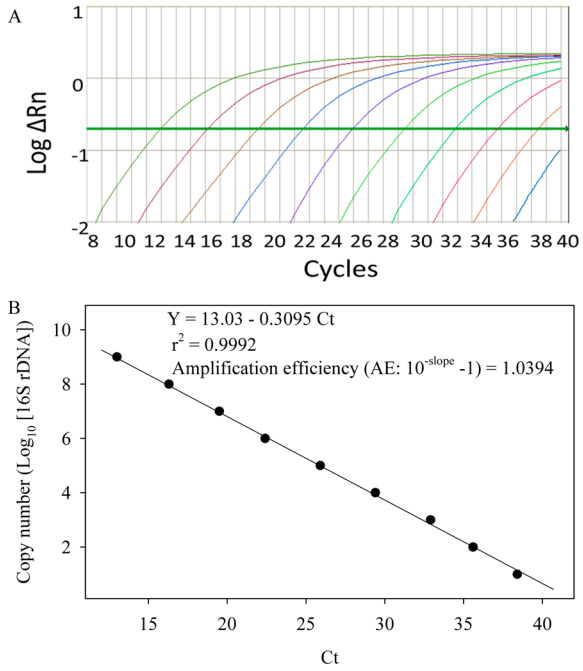

Figure 4. Standard curve of Ct value vs copy number of 16S rDNA serially diluted with plasmid DNA: (A) 16S rDNA ampfication charts in real-time PCR; (B) regression analysis equation. Serial dilution of 16S rDNA: 10^0–10^9 cell per reaction.

$$[16S\ rDNA] = 10^{13.03-0.3095Ct} \quad (1)$$

$Ct \geq 38.5$ is considered to be CLas negative because there was 11% DNA extraction from healthy juice controls that showed Ct values at 38.7–39.7.

Depending on Kim and Wang's[22] results using leaf midribs, there are about three copies of 16S rDNA in CLas bacteria. Thus, the following equation can be used to calculate CLas population

$$[CLas] = \frac{[16S\ rDNA]}{3} = \frac{10^{13.03-0.3095Ct}}{3} \quad (2)$$

Considering sample volume and dilution factors, CLas population in orange juice can be expressed as

$$[CLas]/g\ juice = [CLas] \times \frac{V}{v} \div M \quad (3)$$

where V is the total volume of template DNA (amount of DNA in elution buffer), v is the volume of template DNA per reaction (well), and M is the mass of juice (g) used for DNA extraction. The calculated CLas genome populations in juice are presented in Table 3. The CLas population calculation follows the below equation when Ct = 34.5

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Table 3. Real-Time PCR Results of 16S rDNA and COX in Commercially Processed Valencia and Hamlin Orange Juices[a]

	16S rDNA		COX	16S rDNA/COX	
	Ct	copy number	Ct	ΔCt (16s rDNA-cox)	[COX]/[16S rDNA]
Valencia harvested on 4/21/2011					
healthy	>40[b]	0 b	16.2 a		
asymptomatic	33 a[c]	655 a	14.7 b	18.3 b	322 737 b
symptomatic	32.6 a	872 a	15.4 ab	17.2 a	150 562 a
Hamlin harvested on 12/15/2010					
healthy	39.9[d] c	5 c	15.3 ab	24.6	25 429 504 c
asymptomatic	36.2 b	67 b	16.7 a	19.5 b	741 455 b
symptomatic	30.6 a	3625 a	14.8 b	15.8 a	57 052 a
Hamlin harvested on 02/03/2010					
healthy	38.9 ab	10 b	15.8 b	23.1	8 990 687 c
asymptomatic	30.6 a	3625 a	15.6 b	15.0 b	32 768 b
symptomatic	30.5 a	3893 a	16.5 a	14.0 a	16 384 a

[a]16S rDNA and COX were run separately by simplex qPCR. [b]Ct > 40: not detectable. [c]Values followed by the same letter within the same cultivar harvested at the same time are not significantly different by Tukey's test at the 0.05 level. [d]Average of two replicates. Ct value of the other two replicates was not detectable (>40).

$$[\text{CLas}]/\text{g juice} = \frac{10^{13.03-0.3095\times34.5}}{3} \times \frac{50}{25} \div 7 = 213 = 10^{2.33} \quad (4)$$

where 7 g of juice was used for DNA extraction, total DNA elution was 50 μL, and 2.5 μL of template DNA was used per reaction (well). The results are 213 or $10^{2.33}$ *C*Las cells per gram of juice when Ct = 34.5.

Furthermore, the total *C*Las genome DNA can be expressed as genome DNA mass using Duan et al.'s[54] complete genome sequence (1.23 mb)

$$\text{DNA mass per CLas cell} = \frac{660 \times 1.23 \times 10^6}{6.022 \times 10^{23}} = 1.3481 \times 10^{-15}\ \text{g} = 1.3481\ \text{fg} \quad (5)$$

where 660 is the average mass of dNMP bp and 6.022×10^{23} is Avogadro's constant.

Thus, the *C*Las DNA content in orange juice when Ct = 34.5 is

$$\text{CLas DNA per gram of juice} = 1.348\ \text{fg} \times 213 = 287.12\ \text{fg}\ (1\ \text{fg} = 10^{-15}\ \text{g}) \quad (6)$$

Relative Abundance of *C*Las in Orange Juice. Both 16S rDNA and COX were generally detected using the simplex method in this experiment to avoid interference when multiplex was applied. To avoid variability caused by sample preparation, a relative abundance of 16S rDNA was compared with abundance of COX DNA

$$\Delta\text{Ct} = \text{Ct}_{16\text{S rDNA}} - \text{Ct}_{\text{COX}} \quad (7)$$

With this method, errors carried by different sample preparations and dilutions can be canceled out by the reference gene. Each unit increase at ΔCt value means a 2-fold decrease in the copy number of 16S rDNA genes. The relationship between copy number of 16S rDNA and COX is

$$[16\text{S rDNA}] = \frac{[\text{COX}]}{2^{\Delta\text{Ct}}} \quad (8)$$

Results shown in Table 3 indicate COX DNA is 2^{14}–$2^{24.6}$ times more abundant than that of 16S rDNA in different samples (Table 3). The ΔCt concept makes a comparison between different reports more accurate, because the sample size, extraction efficiency, and dilution factors are no longer an issue. A larger ΔCt represents less *C*Las abundance. As Table 3 shows, juice processed from asymptomatic fruit had larger ΔCt values associated with less severe symptoms in comparison with the symptomatic juice.

Results of qPCR on orange juice processed from healthy, asymptomatic, and symptomatic HLB fruit showed that (1) HLBs juice generally had higher *C*Las populations, (2) HLBa juice had lower *C*Las populations, and (3) some supposedly healthy fruit juice showed Ct values indicating that the juice was 16S rDNA positive, although at a low titer (Table 3). The positive detection for healthy fruit juice indicates that the fruit may have been infected by *C*Las, and sometimes this may have occurred between the last tree testing and the actual time of fruit harvest.

In conclusion, an effective DNA extraction method for qPCR detection of *C*Las in orange juice was developed. Juice samples were mixed with lysis buffer, homogenized using a sonicator, and then incubated with pectinase to hydrolyze pectin. The pH value was adjusted to neutral before proteins were denatured and precipitated by ammonium acetate. After removal of proteins, DNA was precipitated by isopropanol/ethanol and further applied to an elution column-based purification. The role of sonication was to release *C*Las from phloem and resulted in an increase of DNA yield by 86%. The role of pectinase was to eliminate pectin, without which pectin gel traps the DNA. Use of elution column purification removed potential PCR enzyme inhibitors from the DNA extraction solution. With the addition of these steps, *C*Las could be isolated and detected in processed orange juice, which should be of use to the citrus processing industry.

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Notes

The authors declare no competing financial interest.

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