Nested-quantitative PCR approach with improved sensitivity for the detection of low titer levels of Candidatus Liberibacter asiaticus in the Asian citrus psyllid, Diaphorina citri (Kuwayama)

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Article info

Abstract

Candidatus Liberibacter asiaticus (CLas) is a phloem-limited bacterium transmitted by the Asian citrus psyllid (Diaphorina citri). It is the presumptive causal agent of citrus greening disease, also known as huanglongbing (HLB). A critical component of HLB management is the detection of CLas within plant and insect samples to avoid the transport of infected material and to monitor the spread of CLas through citrus groves and psyllid populations. The current method of surveillance is by a presence/absence qPCR assay which utilizes Cq cutoff values to categorize samples as positive, negative, or inconclusive, using the CLas 16S rDNA gene sequence. Upon reviewing the results from routine monthly assays to monitor infection rates in a CLas-positive psyllid colony, it was noted that a large proportion of negative results were being obtained. This observation, coupled with the disjunction between the reported low transmission efficiency of CLas by the psyllid vs. how rapidly HLB has spread, led us to suspect that false-negatives were being obtained using the 16S presence/absence qPCR assay. To investigate this possibility, we developed a nested-qPCR assay with increased sensitivity for CLas. Using this method, we found that the false-negative rate of the presence/absence qPCR assay was greater than 50%, confirming our suspicion and demonstrating that psyllids can harbor titers of CLas below the detection limit of the assay. To investigate if poor qPCR efficiency could be contributing to the high rate of false-negatives, we conducted studies to determine the amplification efficiency, sensitivity, dynamic range and reproducibility of the qPCR assays for CLas 16S rDNA and Wingless (Wg), the D. citri endogenous gene, using plasmid and psyllid DNA templates. Average efficiencies and sensitivity limits of the plasmid assays were 99.0% and 2.7 copies of template for Wg and 98.5% and 2.2–22.1 copies for 16S. Variability in efficiency was significantly greater in psyllid samples for both gene targets compared to the corresponding plasmid assays. Efficiencies as low as 76% were obtained for 16S, suggesting that the sensitivity limit of the assay could be significantly compromised, especially for low-abundant templates. A secondary structure analysis revealed the formation of two stem-loop structures that block the forward and probe binding sites, which could, in part, explain the suboptimal qPCR efficiency of the presence/absence qPCR assay. To circumvent these problems, and thus reduce the rate of false-negatives, an approach to increase the sensitivity of qPCR to circumvent these problems, and thus reduce the rate of false-negatives, is to pre-amplify the gene target using conventional PCR, and use the resulting amplicon as template in qPCR. Known as nested-qPCR, this approach is highly amenable for the detection of disease-causing agents, from viruses to nematodes, and is used regularly to identify these agents in human and plant tissue samples. Examples include the detection of hepatitis C in humans (Santos et al., 2012) and cyst nematodes in potato (Christoforou et al., 2014). Despite the high sensitivity of qPCR, it does have limitations. If the copy number of the template is too low within the sample, or the qPCR reaction is not optimal, adequate amplification may not occur so that the signal from the amplicon is detected above the background fluorescence threshold. In these cases, false-negatives are generated. An approach to increase the sensitivity of qPCR to circumvent these problems, and thus reduce the rate of false-negatives, is to pre-amplify the gene target using conventional PCR, and use the resulting amplicon as template in qPCR. Known as nested-qPCR, this

1. Introduction

Quantitative PCR is an exquisitely sensitive method that, under optimal conditions, can detect a few molecules of template among a complex background of nucleic acids. This sensitivity combined with the high-throughput and cost-effective nature of qPCR, has made it a standard method for diagnostic screening for the presence of disease using characteristic molecular markers associated with disease states, such as cancer (e.g., Hochhaus et al., 2000). This method is also highly

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method has been used to detect low titers of disease-causing agents in humans and plants, such as the detection of *Mycobacterium tuberculosis* in humans (Takahashi and Nakayama, 2006) and *Phytophthora cinnamomi*, the fungus that causes *Phytophthora* root rot in avocado (Engelbrecht et al., 2013).

Microbial plant pathogens, such as viruses, bacteria and trypanosomes, are often transmitted by Hemipteran insect vectors that feed on plant tissue (reviewed in Mitchell, 2004). *Diaphorina citri* (Kuwayama), the Asian citrus psyllid, transmits *Candidatus Liberibacter asiaticus* (*Clas*), a phloem-limited, gram-negative α-proteobacteria and the presumptive causal agent of citrus greening disease (Huanglongbing, HLB). Huanglongbing is a devastating disease of citrus, causing small, asymmetrical, bitter fruit, aborted seeds, and rapid tree decline (Bové, 2006).

At this time, no cure exists, and vector control is a main component of HLB management (Grafton-Cardwell et al., 2013). Rapid screening for *Clas* in psyllid and plant material is imperative in the efforts to control the spread of HLB through inadvertent activities, and qPCR was quickly adopted for the detection of *Clas* in quarantine and field samples. In 2006, Li et al. published a qPCR assay for the detection of the bacterium in plant and insect samples using the *Clas* 16S rDNA gene sequence, and *Wingless* and *Cytochrome Oxidase I* for the detection of psyllid and citrus DNA, respectively. This protocol was then adapted and implemented by the Animal and Plant Health Inspection Service, US Department of Agriculture (APHIS, USDA) to screen vast numbers of quarantine specimens. APHIS publishes a guideline for conducting the high-throughput, standardized presence/absence qPCR assay using cutoff Cq values to categorize samples as positive, negative or inconclusive (USDA, 2012).

Through routine monthly screening to determine infection rates of a *Clas*-positive psyllid colony using the APHIS presence/absence qPCR assay, it was noted that a large proportion of samples were classified as negative or inconclusive, up to 65% and 20% of the total, respectively. Inspection of the amplification plots of the negative samples revealed that some were starting log-phase amplification at the end of the qPCR routine (Fig. A1A). The replicates for these samples were tightly overlaid on one another, suggesting the potential amplification of genuine templates rather than stochastic background noise (Fig. A1B). Other samples crossed the threshold with tight replicates, but did so too late to be considered positive based on APHIS guidelines and were considered negative (Fig. A1C), or inconclusive (Fig. A1D). These observations, coupled with the disjunction between the reported low efficiency by which the psyllid transmits *Clas* (Pelz-Stelinski et al., 2010) vs. the widespread infection rate of citrus with *Clas* in Florida (Gottwald, 2010), led us to suspect that false-negative reporting could be high using the APHIS presence/absence 16S qPCR assay.

Currently, there are no published methods regarding the low-titer detection of *Clas* in either insect or plant tissue, and the lack of a highly sensitive *Clas* detection method may affect disease containment strategies, as well as hinder research efforts (Pelz-Stelinski et al., 2010). Here we report a method to detect low titers of *Clas* within *D. citri*, using the 16S rDNA gene target, with a nested-qPCR approach. In this study, we describe the development and validation of the nested-qPCR method, determine the false-negative rate of the presence/absence qPCR assay, report on the efficiency, dynamic range and sensitivity of the qPCR 16S and *Wingless* qPCR assays using psyllid and *D. citri* DNA templates, and provide evidence that suboptimal qPCR efficiency and secondary structure of template may be contributing to the false-negative reporting of *Clas*.

2. Materials and methods

2.1. *Candidatus Liberibacter asiaticus* — *D. citri* colony

*D. citri* harboring *Candidatus Liberibacter asiaticus* (*Clas*) were obtained from *Clas*-infected *Citrus aurantium* plants maintained in a secure quarantine facility without exposure to insecticides in a greenhouse at 27–28 °C, 60–65% RH, and L14:D10 photoperiod. Monthly sampling conducted throughout 2013 showed that about 50% of *D. citri* individuals obtained from the colony tested positive for *Clas* according to APHIS guidelines (USDA, 2012). Citrus plants were infected with *Clas* by graft-inoculation of healthy ‘Valencia’ *C. aurantium* with infected budwood collected from commercial citrus groves in Immokalee, FL (Collier Co.). Infection was verified via qPCR as described in Pelz-Stelinski et al. (2010).

2.2. DNA isolation from *D. citri*

Genomic DNA was isolated from individual psyllids using the QIAGEN DNeasy Blood & Tissue Isolation Kit (69506; Valencia, CA) followed by the protocol for “Animal Tissues” with modifications. Briefly, per psyllid, 180 μL of ATL buffer and 20 μL of Proteinase K were premixed and 20 μL of the mixture was added to an individual psyllid in a 1.5 ml tube and crushed with a disposable pestle (Kontes, K749520-0090). Another aliquot of 180 μL of the mixture was then added to the psyllid and homogenized with a handheld homogenizer for 5–10 s (Kontes, K749540-0000). Homogenized samples were vortexed for 15 s and placed in a rocking incubator at 56 °C overnight. The next day, 200 μL of AL buffer was added to each tube and vortexed. Mixtures were then incubated at 70 °C for 10 min. After incubation, 230 μL of 100% molecular grade ethanol was added to each sample and vortexed. The mixture was applied to a Spin Column and centrifuged at 16,000 × g for 1 min. The column was washed with 500 μL AW1 and then 500 μL AW2. An additional centrifugation step at 16,000 × g for 1 min was conducted to pull off residual ethanol prior to DNA elution with 35 μL of AE buffer (10 mM Tris, 0.5 mM EDTA, pH 9.0). Eluted DNA was quantified spectrophotometrically using a NanoDrop 2000 (ThermoScientific, Wilmington, DE). To reduce risk of cross-contamination, all surfaces were treated with DNase/RNase solution (Eliminase, O-355-32) and disposable sterile, DNase–RNase-free microcentrifuge tubes and filter tips were used for this and all subsequently described procedures.

2.3. *Candidatus Liberibacter asiaticus* 16S rDNA sequence and primers

GenBank entry DQ778016.1 was used to design primers for the 16S rDNA plasmid for the qPCR standard curve (*Clas*-16S-Temp-F1 and *Clas*-16S-Temp-R1, Table 1; Fig. A2) and the external set of primers for nested-qPCR (*Clas*-16S-Ex-F1 and *Clas*-16S-Ex-R1, Table 1; Fig. A2).

Upon inspection of this GenBank entry and subsequent BLASTn searches, it was noted that the forward qPCR primer designed by Li et al. (2006) contained several mismatches in the forward primer at the 3′ end due to a gap in the original sequence (GenBank: L22532.1). This mismatch was verified upon sequencing of the plasmids for standard curves (see Section 2.5) and was corrected from 5′ TCGAGGCCGTATGCGAACG 3′ to 5′ TCGAGGCCGTATGCGAACGATTACG 3′ (Fig. A2). All primers were designed using Primer3 v. 0.4.0 (Untergasser et al., 2012).

2.4. *D. citri* *Wingless* sequence and primers

To obtain a full-length *Wingless* (Wg) gene sequence for use as a template to plate a gradient for the qPCR standard curve, the partial sequence for the *Wingless* gene (Wg) from *D. citri* (GenBank: AF231365.1) was retrieved from NCBI and used as a BLASTn query against a downloaded and formatted database of the draft *D. citri* genome (v. 1.0; February, 2013) using default parameters. The top hit was found in scaffold 5281.1 and shared 98% identity with the query sequence, with mismatches being ambiguous nucleotides in the query. The scaffold which contained the top hit was retrieved from the database and the Wg coding sequence (CDS) was determined using a BLASTx search in the NCBI nr database. Forward and reverse primers (*Wng-Temp-F1 and Wng-Temp-R1, Table 1, Fig. A3) were designed using Primer3 (Untergasser et al., 2012) to generate a Wg plasmid for the qPCR standard curve as described in Section 2.5.
Table 1

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<td>Clas 16S rRNA</td>
<td>1402</td>
<td>Standard curve template</td>
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<td>69</td>
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</table>

2.5. Molecular cloning and sequencing of the Clas 16S rRNA and Wg plasmids

To generate plasmids for qPCR standard curves, genomic DNA was isolated from five adult D. citri individuals as described above and used as templates in PCR using Takara Taq recombinant Polymerase (Clontech, R001A; Mountain View, CA). Primer, dNTP and MgCl₂ concentrations were 0.5 μM, 200 μM and 1.5 mM, respectively. For the Clas 16S plasmid, the following PCR scheme was used: two cycles of 94 °C for 2 min, 95 °C for 30 s, 63 °C for 45 s, and 72 °C for 90 s followed by 34 cycles of 94 °C for 30 s, 63 °C for 45 s, and 72 °C for 90 s, with a final extension time of 72 °C for 5 min. For Wg, the same scheme was used except for the annealing temperature and extension time, which were 60 °C and 40 s, respectively. PCR reactions were run through a 1.0% agarose gel and all reactions resulted in a single amplicon of the correct size (Table 1). Amplicons were gel purified using Promega’s Wizard® SV Gel and PCR Clean-Up System (A9281; Madison, WI) and were ligated into the cloning vector pGEM-T Easy (A1360). JM109 Escherichia coli cells were transformed with ligation reactions and plated using standard microbiological protocols. Two bacterial colonies for each of 16S and Wg cloning reactions were selected for overnight growth in 3 mL LB/Amp and plasmids were isolated using Promega’s Wizard Plus Miniprep Kit (A7510).

The plasmids were submitted to the University of Florida’s Interdisciplinary Center for Biotechnology Research (ICBR; Gainesville, FL) for Sanger sequencing from both directions using SP6 and T7 universal primers. Sequences were aligned using CLUSTALX 2.1 (Larkin et al., 2007) and inspected for mismatches, which were identified by inspecting respective chromatograms. The two 16S sequences from the plasmids were 99% identical to one another and to the template GenBank entry DQ778016.1 (Table A1, Fig. A2). Sequences from both Wg plasmids were identical to another and to scaffold 5281.1, with a representative sequence submitted to GenBank under accession number KF876339 (Fig. A3). All inserts were oriented in the pGEM-T Easy vector such that the PstI restriction enzyme site used to linearize the plasmids was located at the 3’ end.

2.6. Quantitative PCR

Quantitative PCR was conducted with an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) based on a multiplex qPCR assay described previously with modifications (Pelz-Stelinski et al., 2010; USDA, 2012). Briefly, the Wingless gene from D. citri was used as an endogenous control for psyllid DNA and 16S rDNA was used for the detection of Candidatus Liberibacter asiaticus. The qPCR reaction volume was 20 μL containing 0.1 μM of each primer and probe (Table 1) in 1× TaqMan Universal PCR Master Mix containing ROX™ as the passive internal reference dye (Applied Biosystems, 4304437). The probe for Wg was labeled with 4,5-dichloro-dimethoxy-fluorescein (JOE™) and 16S with 6-carboxy-fluorescein (FAM™). Standards and samples were added to individual wells of the qPCR plate in a 2 μL volume and 18 μL of the master mix was added over the sample to wash the entire mixture to the bottom of the well. Other parameters, such as amount of template, are indicated in relevant sections below.

All qPCR reactions were performed in triplicate except for the APHIS presence/absence screening assays to identify negative and positive samples, which were performed in duplicate. All plates included a non-template control in triplicate with 2 μL Qiagen Elution Buffer (EB, 10 mM Tris, pH 8.5). Plates were prepared in a sterile, biological PCR laminar flow cabinet. The qPCR scheme for all qPCR reactions was 95 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, with fluorescence data collected at the last step. Sample amplification plots were inspected using the Applied Biosystems 7500 software (v. 2.0.1) and raw data were exported as a tab-delimited file and analyzed in Microsoft Excel (2013).
Plasmids were submitted for Sanger sequencing at the University of Florida ICBR Genomics Core (Gainesville, Florida) using the T7 universal primer. Sequences were aligned and evaluated with CLUSTALX 2.1 (Larkin, et al., 2007).

2.8. Optimization of nested-qPCR

Because the 1.5 dilution resulted in very low Cq values, the optimal dilution factor was determined by diluting conventional PCR reactions from two samples; one positive and one negative from the qPCR presence/absence assay, in a ten-fold serial dilution from 10−1 to 10−8 and used as templates in nested-qPCR. A positive sample was run to estimate the lower Cq boundary of the range in each dilution series that might be observed. It was determined that a dilution of 10−2 to 10−3 produced average Cq values of 4.1 and 6.7, and 13.9 and 17.3, for positive and negative samples, respectively, from the initial qPCR screening assay (Fig. A4). Although the dilution of 10−2 still resulted in low Cq values for the positive sample, the 10−2 dilution produced Cq values high enough to allow the instrument to accurately set baseline fluorescence, and thus was adopted in the subsequent assay for determining an estimate for false-negative rates.

2.9. Estimates of the false-negative error rate in the 16S presence/absence qPCR assay

DNA isolated from psyllids for routine monthly screening for infection rate of a Clas-positive colony was used to determine the rate of false-negatives in the presence/absence qPCR assay as compared to the nested-qPCR assay. DNA samples from five months were analyzed, September through November, 2013; and January through February, 2014. Twenty psyllids were screened for each month except for November, for which 18 psyllids were screened. An average of 65 ng was used in a presence/absence qPCR assay to determine initial estimates for Clas infection. One microliter of each negative sample was used in conventional PCR as described above. Reactions were diluted 1:1000, including the NTC from the conventional PCR, and 2 μl of each dilution was used as template in nested-qPCR.

2.10. Performance evaluation of the Wg and 16S qPCR assays

To investigate the efficiency and sensitivity of the Wg and Clas 16S TaqMan qPCR assays, calibration curves were generated from serial dilutions of linearized Wg and 16S plasmids. One microgram of each plasmid was digested with PstI restriction endonuclease (Promega, R6111) in a 20 μl reaction volume and subsequently purified using a MinElute Reaction Cleanup Kit (Qiagen, 28204). Samples were eluted in 10 μl EB. Quantification of purified, linearized plasmids was conducted spectrophotometrically with a NanoDrop 2000. Two microliters of each purified plasmid was run through a 1.0% agarose gel to confirm complete digestion and the presence of only one PstI restriction endonuclease recognition site within each plasmid. Seven, ten-fold serial dilutions were made with each plasmid ranging from 5 pg/μl to 50 μg/μl. The amplification efficiency of Wg and 16S was also determined for genomic DNA isolated from individual psyllids using four, ten-fold serial dilutions ranging in concentration of 20 ng/μl to 200 pg/μl. To reduce potential effects on efficiency due to a buffer concentration gradient across dilutions, EB was used make serial dilutions. Five independent calibration curves were generated for each plasmid, and 15 Wg and 16S sample curves were generated from DNA isolated from independent psyllids previously been shown to be positive for Clas. Averaged raw Cq data were plotted against log input DNA (log pg or log ng) to determine slope of each regression line. Quantitative PCR efficiencies were calculated using the basic equation: efficiency = 1 + 4 [1−1/[slope]]. Molecular weights of plasmids were calculated using Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/dna_mw.html). An analysis of deviance was conducted to determine statistical differences between plasmid and sample calibration curves using GenStat, 15th Edition, Vsn International.

2.11. Secondary structure prediction of Wg and 16S qPCR amplicons

Predictions of secondary structures of Wg and 16S amplicons were determined using the DINAmeR Web Server (http://mfold.rna.albany.edu/?q=DINAmeR; Markham and Zuker, 2005) using the following parameters: Molecule type, DNA; temperature, 60 °C; [Na+] 50 mM; and [Mg2+] 1.5 and 4.0 mM. The concentration of magnesium is proprietary in the TaqMan Universal Master Mix but is reported as being between 1.5 and 4.0 mM, thus secondary structure was determined at these two concentrations.

3. Results

3.1. Validation of external primers for nested-qPCR and verification of the specificity of the nested-qPCR 16S amplicon

DNA samples from 20 psyllids collected in September 2013 from a Clas positive colony were evaluated for Clas in a presence/absence qPCR assay. Results were categorized as positive, negative or inconclusive according to APHIS guidelines (USDA, 2012) as described above (Fig. 1 and Table A2). The Cq values obtained for the endogenous control, Wg, for all 20 samples ranged between 22.8 and 24.3 with an average of 23.5 (SD 0.4). No amplification signals were detected for Wg or 16S in the non-template control (NTC). Out of 20 samples, 11 were positive for 16S, with Cq values ranging from 18.3 to 26.9, and four samples were classified as inconclusive (Cq 32.1, 34.3, 35.5 and 36.2). Five samples were negative, four that were undetected and one with a Cq value too high to be considered inconclusive (39.0). Based on the presence/absence qPCR assay, the breakdown of infection was: 55% positive, 20% inconclusive, and 25% negative (Fig. 1). From this screening, eleven

Fig. 1. Clas infection rate of psyllids surveyed by month as determined by A. positive/negative qPCR. Positive = Cq ≤ 32; Inconclusive = 32 < Cq < 37; negative = Cq > 37; and B. nested-qPCR, n = 20 except for November, which n = 18. Total: summarized total for all five months.
samples were selected for pre-amplification in conventional PCR and subsequent nested-qPCR to validate the nested primers and test the assay; five negative samples; two inconclusive samples (C 34.3 and 35.5); and four positive samples (C 18.3, 19.5, 25.7, 25.8). Ten samples tested positive with positive Cq values ranging from 2.3 to 10.2. One of the negative samples, Sept 1c, resulted in a nested-qPCR Cq value of 38.7, which raises questions about appropriate cutoffs for Cq values in this assay that remain to be decided, but for the purposes of this study, this sample was considered to be negative (Table A2). Importantly, no signal was detected in the conventional PCR or nested-qPCR NTC, supporting the notion that contamination did not play a role in observed results. No signal was observed using DNA isolated from the sharpshooter, C. costalis, as a template. The sequences from all eleven amplicons were 100% identical to one another and to the expected sequence for 16S as represented by GenBank entry DQ778016.1 (Fig. 2).

The remaining samples that were initially categorized as inconclusive were tested in a nested-qPCR assay and found to be positive, for a final infection rate of 95% (Fig. 1).

3.2. Estimates of the false-negative error rate reported by the presence/absence qPCR assay

Comparison of the two methods, the presence/absence qPCR and nested-qPCR, was conducted over the course of five months starting with the data collected in September 2013. Wg was detected in all 98 samples with an average Cq of 24.9 (SD 1.0; Table A2). Infection rates of 35, 45, 44, 35 and 35% were determined by the presence/absence qPCR assay for the months of September, October and November 2013, and January and February 2014, respectively (Fig. 1A). Collectively, 42 samples out of 98 were positive by APHIS guidelines, with a final infection rate of 42.9%. Out of the remaining 56 samples, 8 were inconclusive and 44 were negative (Fig. 1A, Table A1). In comparison, the infection rate as determined by nested-qPCR was 95% for September, 94.4% for November, and 100% for October, January and February. The final infection rate for all psyllids sampled across the five months using nested-qPCR was 97.9% (Fig. 1B, Table A2), resulting in an overall false-negative rate of 45.9.

An unexpected observation was made in analyzing psyllid samples collected over the course of several months, and that is the rate of infection that was detected by the presence/absence qPCR assay dropped gradually as the months progressed into the winter (Fig. 1A), presumably as an indicator of dropping Clas titers.

Whether this observation is biologically relevant or is an artifact of rearing insects in an artificial environment is unknown, but is consistent with the finding that Clas titers within the insect host is affected by temperature (Kingdom Gibbard et al., unpublished results).

3.3. Evaluation of qPCR assays: dynamic range, sensitivity and efficiency

The dynamic range, sensitivity and efficiency of the Wg and 16S qPCR assays were evaluated using calibration curves generated with ten-fold serial dilutions of linearized plasmid templates. These curves were then compared to the Wg and 16S calibration curves generated with 10-fold serial dilutions of genomic DNA isolated from individual psyllids harboring Clas. Five independent trials were conducted for plasmid DNA and 15 for psyllid DNA, the results of which are summarized in Fig. 3 and Tables A3 and A4. The dynamic range of the Wg plasmid qPCR assay spanned seven orders of magnitude, with an average R² value of 0.993 (SD 0.002), with the reproducibility between trials relatively high (Fig. 3A). The estimated minimum sensitivity of the assay was 2.7 starting molecules of template (Table A3). The range of efficiency for the Wg linearized plasmid across the five trials was 97.4–103%, with an average of 99.2% (SD 2.3%). The 16S plasmid assay was less robust than that for Wg (Fig. 3B). The average R² value of calibration curves across the five trials was 0.983 (SD 0.019), with two trials falling below the desired R² cutoff of 0.99. The assay spanned seven orders of magnitude in two trials (sensitivity: 2.21 molecules; data not shown) and six in the remaining three (sensitivity: 22.1 molecules) (Table A3). Efficiencies ranged from 93.4 to 103.2% with an average amplification efficiency of 98.9% (SD 3.6%). While the overall performance of the 16S assay was well within the guidelines for qPCR, it is not as sensitive as Wg (Fig. 3A vs B; Table A3). Maximal sensitivity is particularly important given that the Clas DNA template will be in low abundance relative to that of the host DNA. Low abundant templates are particularly sensitive to stochastic error that is inherent in every qPCR assay, and run the risk of being underestimated or undetected, even in well-performing assays. Any factor that reduces qPCR efficiency or sensitivity will exacerbate this phenomenon. In many cases, plasmid calibration curves perform better than those made from samples, with two main causes being non-specific binding of primers to non-target DNA, and inhibitory contaminants that are co-isolated during DNA purification. Therefore, it was not unexpected that the parameters of the calibration curves made using psyllid DNA were not as robust as the Wg and 16S those made from plasmid DNA (Fig. 3C and D vs Fig. 3A and B; Tables A3 and A4). The variability of the slopes, which correspond directly to qPCR efficiency (see formula in Materials and methods section), of the sample calibration curves were significantly greater than that of the plasmids (Wg: F 0.20 72.30, p = 0.001; 16S: F 0.20 20.50, p = 0.006). The Wg qPCR efficiencies for psyllid DNA samples ranged from 73.4 to 101.2% with an average efficiency of 91.1% (SD 7.8%) and nine out of the 15 standard curves had efficiencies that fell within the accepted range of 90–110% (Fig. 4). The average R² value for all curves was 0.996 (SD 0.004), with all but two making the 0.99 cutoff (Table A4). The corresponding 16S assays performed less optimally. Efficiencies for nine out of the 15 calibration curves for 16S from the same psyllid samples fell outside of the accepted range (Fig. 4, Table A4), four of which had nonsensical efficiencies of 150% and above (Table A4). The corresponding Wg standard curves from the same psyllid samples for these four assays were as expected and within acceptable parameters for qPCR assays (Figs. 3 and 4, Table A4), with low standard deviations between technical replicates (≤0.5; data not shown), showing that these results were not due to error in serial dilution. This, coupled with normal reactions for 16S positive controls in the assay that rule out errors made with master mixes or degradation of primers and/or probes, suggests that stochastic error or other unknown factor, to which the 16S assay is particularly sensitive, contributed to these aberrant results, rather than technical error (Fig. 3D; Table A4). The remaining six 16S efficiencies ranged from 76.0 to 100.25% with an average of 91.1%.

![Fig. 2. Candidatus Liberibacter asiaticus 16S sequence alignment of nested-qPCR products with reference 16S RNA sequence CP001677.5.](image-url)
95.9% (SD 4.2%). It is interesting to note that in all but two of the 16S assays, it would require greater than 50 cycles of qPCR to detect 22 molecules of template, the highest reliable dilution determined in the corresponding 16S assay using plasmid (Table A3). All but four regression lines met the required cutoff of 0.99 with an overall average R² of 0.997 (SD 0.004; Table A4). As would be expected for samples with varying titers of CaLas, the intercept of the calibration curves varies widely as this parameter is directly affected by starting copy number of template. The most striking result is that in some cases, the efficiencies of 16S vs Wg from the same psyllid sample differed significantly, suggesting that the 16S assay may be more sensitive than the Wg counterpart to inherent differences between DNA isolations. As stated above, contamination co-isolated with the DNA such as salt, buffer or carbohydrates, or other contaminant can negatively impact qPCR efficiency. However, no correlation was found between the measured A₂₆₀/A₂₃₀ ratio and efficiency for either gene target (data not shown). Interestingly, in some cases, the standard deviations between technical replicates were well below the 0.3 cutoff for one target, while exceeding the cutoff for the other target from within the same sample, again suggesting that stochastic error, rather than pipetting or other technical error, contributed to variation across replicates (data not shown).

3.4. Secondary structure predictions of qPCR Wg and 16S amplicons

To determine if secondary structure could potentially contribute to greater variability in efficiencies of the Wg and 16S qPCR assays, structures and their thermodynamic parameters were predicted using the DINamelt Webserver (Markham and Zuker, 2005). The thermodynamic parameters for Wg were: ΔG = 0.1 kcal/mol, ΔH = −18.9 kcal/mol, ΔS = −56.9 e.u., Tm = 59.1 °C; and ΔG = −0.1 kcal/mol, ΔH =...
with absolute certainty that all negatives are genuinely negative, but this is the screening of budwood to be certi-

The insect or plant of 538 methods to control or combat the disease, such as treatments to clear 537

tion. False-negatives are also problematic in the evaluation of new 534

and biochemical questions such as: the localization of 526 consequences. For example, the use of an assay with high rates of false-

change the outcome and the extra step to investigate them would be 524
to address that question. In many cases, a few false-negatives will not 520

to simulate of suf- 516

predictions of: \( \Delta G = -1.8 \text{kcal/mol}, \Delta H = -94.4 \text{kcal/mol}, \Delta S = -278.0 \text{e.u.}, T_m = 66.5 \degree C; \) and \( \Delta G = -2.4 \text{kcal/mol}, \Delta H = -94.4 \text{kcal/mol}, \Delta S = -276.1 \text{e.u.}, T_m = 68.8 \degree C \) for 1.5 and 4.0 mM Mg\(^{2+}\), respectively. The secondary structure for 16S contained two loops at the 5’ end of the amplicon; one blocking the binding site for the forward primer and the second blocking the binding site for the probe (gray shading, Fig. 5B). These results suggest that secondary structure could have a negative impact on 16S qPCR amplification efficiency.

4. Discussion

Here we describe a nested-qPCR assay that can be used in conjunc-
tion with the Clas 16S presence/absence qPCR assay to reduce the num-
ber of false-negatives obtained when using the presence/absence assay 520 alone. It is simple, inexpensive, and can be used in series with existing 522 assays without disrupting workflow. The decision to employ the com-
panion assay will depend on the question being addressed, and whether 524 or not false-negatives will affect the interpretation of the data collected 526 to address that question. In many cases, a few false-negatives will not 528 change the outcome and the extra step to investigate them would be 524 wasted effort. Examples include the detection of one positive sample 528 within a batch that calls for the destruction of the entire batch, or mak-
ing the determination if any insects within a colony test positive for 524 Clas. However, there are cases where false-negatives could have serious 526 consequences. For example, the use of an assay with high rates of false-

negatives is inappropriate for addressing complex biological, molecular 528 and biochemical questions such as: the localization of Clas within plant 524 and insect tissues, the elucidation of transmission rates given certain 524 conditions, or the determination of what tier levels constitute an infec-
tion. False-negatives are also problematic in the evaluation of new 526 methods to control or combat the disease, such as treatments to clear 528 the insect or plant of Clas, or the identification of resistant cultivars. 528 False negatives are of the most serious consequence in instances when 524 the tolerance of a disease-causing agent is zero. A relevant example of 524 this is the screening of budwood to be certified as free of viruses and 526 other graft-transmissible diseases. No method of detection can say 528 with absolute certainty that all negatives are genuinely negative, but

for the purposes described above, the method of detection must be opti-
mized to provide the greatest sensitivity possible, i.e., optimized to 525 provide the lowest rate in false-negative reporting achievable. As seen 525 in this study, the rates of false-negative reporting can be significantly 526 high in the presence/absence qPCR assay — up to more than half of 526 the samples tested. We propose that two characteristics inherent in 526 the 16S target contribute to problem of underestimation made by this 529 assay: low abundance and sensitivity to suboptimal conditions. Both 531 Wg and 16S showed greater variability in performance using DNA isolat-
d from psyllids over that observed for plasmid DNA, in some cases 533 performing below acceptable parameters. For Wg that is not as prob-

lematic because this gene target is in abundance. However, 16S can 535 be in low abundance, especially in samples where bacterial titer is low. 535 It is well demonstrated that the stochastic error inherent in any qPCR 537 assay, even one that has been developed to perform optimally, has 538 a greater effect on the amplification of low-abundant targets (<1000 cop-
es) than those in greater copy numbers, and that the lower the starting 531 copy number, the less likely the abundance will be reflected in the final 535 analysis of the qPCR (Bustin and Nolan, 2004; Kar rer et al., 1995). The 537 implication of this is that for some low-abundance templates, the accu-
mulation of sufficient product for signal detection above the fluoresce-

nt background noise might not occur, and template within samples goes 543 undetected. Any factor that negatively impacts efficiency will magnify 545 this limitation of qPCR. Several observations made in this study suggest 545 that the efficiency of the 16S assay is variably affected by some compo-
nent present DNA samples isolated from host tissue. First, the 546 Wg and 16S qPCR assays perform well under the conditions of pure template 550 in highly purified conditions. This demonstrates that overall, the qPCR 552 assays are optimal for the basic parameters of the reaction, such as mag-

esium, primer and probe concentrations. However, it was noted that 554 the 16S assay is on average an order of magnitude less sensitive than 556 the Wg counterpart. In using calibration curves with psyllid DNA, differ-
ces in performance between the Wg and 16S start to diverge further. 557 Most of the Wg assays performed within acceptable efficiency range for 557 qPCR assays, the majority of the 16S assays did not, with 60% falling out-
side the acceptable range of 90–110%. In four cases, calculated efficien-
cies for 16S were nonsensical values of greater than 150%, while the 557 corresponding Wg assays were normal; suggesting something within 559 the isolated DNA sample is affecting 16S more profoundly than Wg. 560 In one case, \(C_q\) values for 16S were about 24.5 for all four, ten-fold serial di-
lutions of the psyllid sample. This was not an error in dilution or pipet-
ting because the serial dilution for the Wg counterpart from the same 564 psyllid sample was normal with a calculated \(C_q\) value of 24.5. In most cases, there was a lack of correlation in the efficiencies for most 566 of psyllid samples between Wg and 16S. In summary, 16S qPCR assay 568 is not as sensitive or consistent in its performance as compared to the 568 Wg counterpart, and can demonstrate aberrant behavior using psyllid 570 DNA as template. Our current hypothesis is that contaminants 572

![Fig. 5. Predicted secondary structures of A. Wg and B. 16S amplicons at 60 °C. Predicted structures were the same for both 1.5 and 4.0 mM Mg\(^{2+}\). Gray shading are primer (5’ and 3’ ends) and probe binding (center) sites.](image)

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co-isolated with sample DNA are contributing to these results, which is consistent with the observations in other systems (e.g., Hargreaves et al., 2013). In addition, the two relatively stable secondary structures that are thermodynamically favorable at the thermocycling temperatures used in the 16S qPCR assay may contribute to less-than-optimal amplification as these stem/loop structures may block the primer binding site of the forward primer. Secondary structures formed in an amplicon are known to cause reduction of PCR efficiency (McDowell et al., 1998). While the utility of 16S in PCR-based methods for the discovery, identification and detection of pathogens are undeniable (for a historical overview, see Sontakke et al., 2009), the propensity to form secondary structure, on which its biological function depends, needs to be considered when designing primers. It is also possible that the introduction of host DNA provides template for non-specific binding of the Clas primers, thus forming non-specific products, resulting in decreased efficiency of the qPCR reaction. Most likely, the observations made in this study are a result of a complex combination of underlying factors, and it is not surprising that other gene targets for Clas detection are now being used (Ananthakrishnan et al., 2013). However, we chose to develop a nested-qPCR assay to be compatible with previous studies conducted by our group that utilized the 16S assay, and for researchers who are not able to shift to different gene targets for various reasons. While other methodologies with even greater sensitivity than qPCR are available and are being used for screening for disease-causing agents, such as Droplet Digital™ PCR (ddPCR™, Bio-Rad; Carlsbad, CA; White et al., 2012), they are not currently mainstream. In addition, we are evaluating methods to enrich for bacterial DNA isolation from host tissue, both of plant tissue as well. We have used this method to screen plant tissue for Candidatus Liberibacter asiaticus in the... J. Microbiol. Methods (2014), http://dx.doi.org/10.1016/j.mimet.2014.04.007.

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