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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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ABSTRACT

Candidatus Liberibacter asiaticus (*C*Las) is a phloem-limited bacterium transmitted by the Asian citrus psyllid, *Diaphorina citri*, and the presumptive causal agent of citrus greening disease. The current method of detection for CLas within plant and insect samples is by a presence/absence qPCR assay using the CLas *16S* rDNA gene target. Although qPCR is highly sensitive, low bacterial titers or suboptimal qPCR conditions can result in false-negatives. Using a nested qPCR assay, we determined the false-negative rate of the *16S* presence/absence qPCR assay was greater than 50%. Studies to determine the performance parameters of the qPCR assays for CLas *16S* and *Wingless* (*Wg*), the *D. citri* endogenous gene, using plasmid and psyllid DNA, revealed suboptimal and variable performance of the *16S* assay in psyllid samples. Average efficiencies and sensitivity limits of the plasmid assays were 99.0% and 2.7 copies of template for *Wg*, respectively, and 98.5% and 2.2-22.1 copies for *16S*, respectively. Variability in efficiency was significantly greater in psyllid samples for both gene targets compared to the corresponding plasmid assays, and efficiencies as low as 76% were obtained for *16S*. A secondary structure analysis revealed the formation of two stem-loop structures that block the forward and probe binding sites in the *16S* template, which could hinder amplification. In summary, our results suggest that suboptimal qPCR efficiency is not uncommon for the *16S* presence/absence qPCR assay, which combined with lowCLas titers in some samples, could contribute significantly to the under-reporting of CLas infection in psyllid and plant samples.

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 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 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 cinnamoni*, 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

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(*C*Las),[1] a phloem-limited, gram-negative α-proteobacteria and the presumptive causal agent of citrus greening disease (Huanglongbing, HLB).[2] 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 *C*Las 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 *C*Las in quarantine and field samples. In 2006, Li et al. published a qPCR assay for the detection of the bacterium in plant samples using the *C*Las *16S* rDNA gene sequence, and *Cytochrome Oxidase I* as the citrus endogenous gene. Shortly thereafter, an assay for *C*Las detection in psyllids was developed using *Wingless* as the endogenous control for *D. citri* (Li et al., 2008). These assays were 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 C_q values to categorize samples as positive, negative or inconclusive (USDA, 2012). Through routine monthly screening to determine infection rates of a *C*Las-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 *C*Las (Pelz-Stelinski et al., 2010) vs. the widespread infection rate of citrus with *C*Las 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 *C*Las in either insect or plant tissue, and the lack of a highly sensitive *C*Las 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 *C*Las 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 plasmid and *D. citri* DNA templates, and provide evidence that suboptimal qPCR efficiency and secondary structure of template may contribute to the false-negative reporting of *C*Las.

2. Materials and methods

2.1. Candidatus Liberibacter asiaticus — D. citri colony

D. citri harboring *Candidatus* Liberibacter asiaticus (*C*Las) were obtained from *C*Las-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 *C*Las according to APHIS guidelines (USDA, 2012). Citrus plants were infected with *C*Las 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).

[1] *C*Las: *Candidatus* Liberibacter asiaticus.
[2] HLB: Huanglongbing.

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) using 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 set 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, 04-355-32) 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′ TCGAGCGCGTATGCAATACG 3′ to 5′ TCGAGCGCGTATGCGAATAC 3′ (Fig. A2). All primers were designed using Primer3 *v*. 0.4.0 (Untergrasser 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 generate a plasmid 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 (Untergrasser et al., 2012) to generate a *Wg* plasmid for the qPCR standard curve as described in Section 2.5.

2.5. Molecular cloning and sequencing of the CLas 16S rDNA 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 $MgCl_2$ concentrations were 0.5 μM, 200 μM and 1.5 mM, respectively. For the