Developing dPCR For Detecting Phytoplasmas in Palms

Brian Bahder, UF/IFAS Fort Lauderdale REC, bbahder@ufl.edu

ABSTRACT

Phytoplasmas are an economically important group of plant pathogens that negatively impact a wide variety of plants in agricultural and natural ecosystems. In Florida, palms are essential elements in the nursery and landscaping industries that suffer from diseases caused by phytoplasmas that are related to each other but are classified in two different subgroups, 16SrIV-A and 16SrIV-D. In this study, a TaqMan assay was developed for digital PCR (dPCR) to detect both palm-infecting phytoplasmas found in Florida. When compared to real-time PCR assays and nested PCR assays, dPCR was capable of detecting the phytoplasmas at much lower concentrations than was possible by using real-time PCR and nested PCR. Due to sequence identity of primer annealing regions across diverse phytoplasmas, the assay is likely to be successful for detection of a wide variety of phytoplasmas. The increased sensitivity of this dPCR assay over real-time PCR will allow for earlier detection of phytoplasma infection in palms, as well as for screening of salivary glands of candidate insect vector species. These advantages should aid timely management decisions to reduce disease spread and rapid determination of phytoplasma transmission by vectors.

OBJECTIVE

To develop a highly sensitive molecular tool for detection of palm-infecting phytoplasmas for use with dCPR.

METHODS

Isolates of LY and LB used in this study were selected from samples stored at the Fort Lauderdale Research and Education Center (FLREC) that had previously tested positive for the 16SrIV-A and 16SrIV-D phytoplasmas and were confirmed by sequencing. The LY isolate was obtained from an infected *C. nucifera* sampled at FLREC (Isolate ID: LYJT-EF) and the LB isolate was obtained from an infected *S. palmetto* sampled in Manatee County, FL (Isolate ID: Sab1+C). A Sabal palm (Isolate ID: Sabal4) that had previously tested negative for phytoplasma infection was used as a healthy control. The total DNA extractions for each of the isolates tested were serially diluted nine times (10X dilution factor), using Qiagen Buffer AE allowing for 10 different concentrations of each isolate. One microliter of the total DNA and each of the dilutions for all isolates was quantified using the Qubit® dsDNA BR Assay Kit with a Qubit ® Fluorometer per the manufacturer's protocol.

To obtain the primers and probe necessary for dPCR, sequence data for the entire 16S gene for the 16SrIV-D phytoplasma (Accession number AF434989) was uploaded into Thermo-Fishers web-based portal using the custom copy number option. The region selected was aligned with the same region of the 16SrIV-A phytoplasma to ensure sequence identity between the two strains at the primer and probe regions. This region was also aligned with all other subgroups of the 16SrIV taxonomic group available on GenBank, as well as the A subgroup from each of the existing 16Sr groups and two outgroups, *Fibrobacter succinogenes* and *Staphylococcus aureus*. This alignment process was to provide insight to the range of phytoplasmas that this assay could detect and to ensure that it would not amplify non-phytoplasma bacterial DNA. The probe was labeled with the 5' reporter dye, FAM and a 3' quencher, NFQ. Digital PCR assays were performed in 14.5 µl, as per the manufacturer's instructions. Each reaction was comprised of 6 µl of DNA template, 7.25 µl of QuantStudio™ 3D Digital PCR Master Mix v2, 0.725 µl of the custom copy number TaqMan assay, and 1.525 µl of water. Reaction mixtures were loaded on QuantStudio™ 3D Digital PCR 20K Chips v2 using the QuantStudio™ 3D Digital PCR Chip Loader and run on a ProFlex Base PCR System. Thermal cycling conditions consisted of an initial denaturation stage of 95°C for 10 min., followed by 35 cycles of denaturation at 95°C for 30 sec., annealing at 60°C for 30 sec., and extension at 72°C for 30 seconds, and a final extension of 5 min. at 72°C. End-point fluorescent data were collected on a QuantStudio™ 3D Digital PCR System and analyzed using the QuantStudio™ AnalysisSuite Cloud Software.

RESULTS

The total DNA measured for each dilution for both LB and LY phytoplasmas is presented in Table 1. The region selected for primer design was a 73 bp product, starting at nucleotide 872 of the 16S gene and ending at nucleotide 945. Based on the sequence alignment of the primer regions and the probe annealing location, there is 100% identity among all strains of the 16SrIV phytoplasmas recorded from diseased palms (Fig. 1) of the target region in the dPCR assay. Also, both the primer and probe regions share a high level of identity with other 16Sr group phytoplasmas outside of the 16SrIV group (Fig. 1). The primer sequences for the TaqMan assay are 5'-CCTGAGTAGTACGTACGCAAGT-3' (forward) and 5'-CAACATGATCCACCGCTTGTG-3' (reverse). The probe sequence is 5'-FAM-TCCCGTCAATTCCTTTAAGTTTC-3'. The probe is the reverse compliment to the region highlighted in Figure 1, which is in a 5' to 3' direction.

Table 1. Total DNA measured in all serial dilutions for both the 16SrIV-A and 16SrIV-D isolates and the healthy control.

	Quantity (ng/µl)		
Dilution	A	D	Control
1	464	48.5	32.5
1e-1	48	<0.010	<0.010
1e-2	2.52	<0.010	<0.010
1e-3	<0.010	<0.010	<0.010
1e-4	<0.010	<0.010	<0.010
1e-5	<0.010	<0.010	<0.010
1e-6	<0.010	<0.010	<0.010
1e-7	<0.010	<0.010	<0.010
1e-8	<0.010	<0.010	<0.010
1e-9	<0.010	<0.010	<0.010

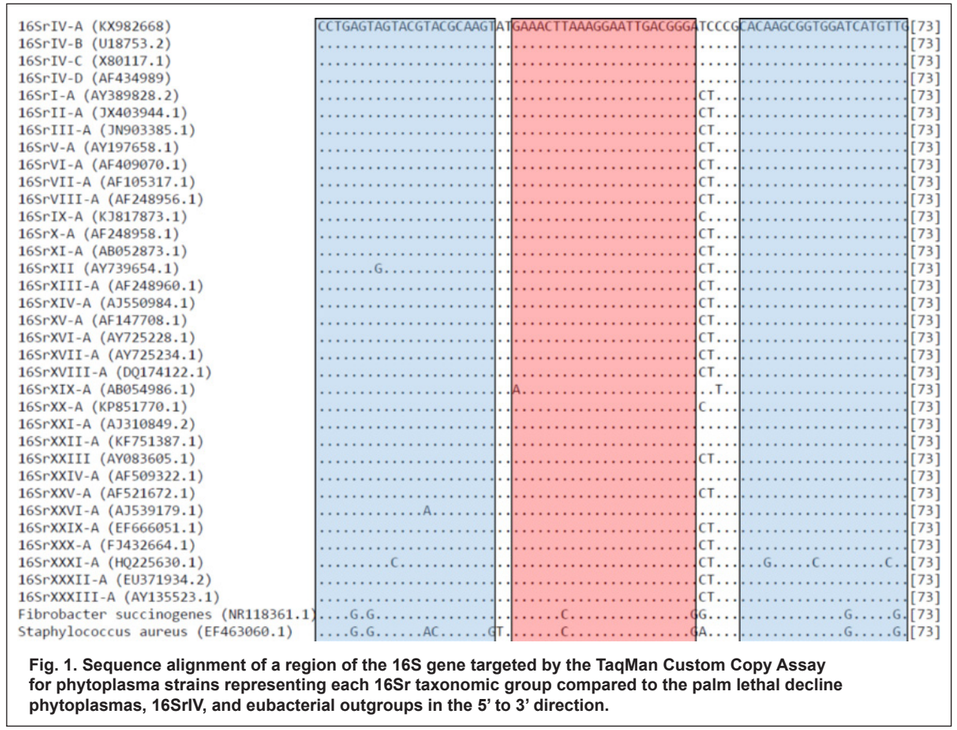

Fig. 1. Sequence alignment of a region of the 16S gene targeted by the TaqMan Custom Copy Assay for phytoplasma strains representing each 16Sr taxonomic group compared to the palm lethal decline phytoplasmas, 16SrIV, and eubacterial outgroups in the 5' to 3' direction.

The TaqMan assay successfully amplified the target region for both the 16SrIV-A phytoplasma (Table 2) and for the 16SrIV-D phytoplasma (Table 3). The original extract for the 16SrIV-A isolate had a high enough titer of phytoplasma to fully saturate the dPCR chip, resulting in amplification success of all wells passing the assay quality threshold (Fig. 2). Additionally, the first serial dilution for the 16SrIV-A isolate was too concentrated and also yielded a fully saturated chip with all wells passing threshold exhibiting amplification (Fig. 2). The optimal reaction for 16SrIV-A was the $1e^{-2}$ (1% diluted from original extract) dilution with the pathogen being detectable at the $1e^{-6}$ dilution (0.0001% diluted from original extract) (Fig. 2). This reaction allowed for the most accurate estimate of phytoplasma amplicon target copies/µl; estimates of copies/µl in the original extract could not be calculated from the original extract itself or the $1e^{-1}$ dilution due to complete saturation of the chip with target DNA. The $1e^{-7}$, $1e^{-8}$, and $1e^{-9}$ dilution reactions also did not provide estimates of copies/µl for the original extract due to no positive reactions on the chips. The $1e^{-3}$, $1e^{-4}$, $1e^{-5}$, and $1e^{-6}$ all provided an estimate of the copies/µl of the original extract (Table 2) but were not as accurate as the $1e^{-2}$ reaction and with accuracy decreasing as sample became more diluted (Table 2).

Table 2. Digital PCR data for the serial dilution of the 16SrIV-A isolate.

Dilution	Total Wells	(+) Wells	(-) Wells	Copies/µl	Precision
1	16,887	16,887	0	N/A	N/A
1e-1	17,667	17,667	0	N/A	N/A
1e-2	17,791	9,612	8,179	1,029.20	1.48%
1e-3	16,212	1,421	14,791	121.55	5.34%
1e-4	17,596	168	17,428	12.71	16.33%
1e-5	17,538	15	17,523	1.13	65.88%
1e-6	16,816	6	16,810	0.47	122.59%
1e-7	17,122	0	17,122	N/A	N/A
1e-8	17,912	0	17,912	N/A	N/A
1e-9	16,545	0	16,545	N/A	N/A
Healthy	17,544	0	17,544	N/A	N/A
Water	18,909	0	18,909	N/A	N/A

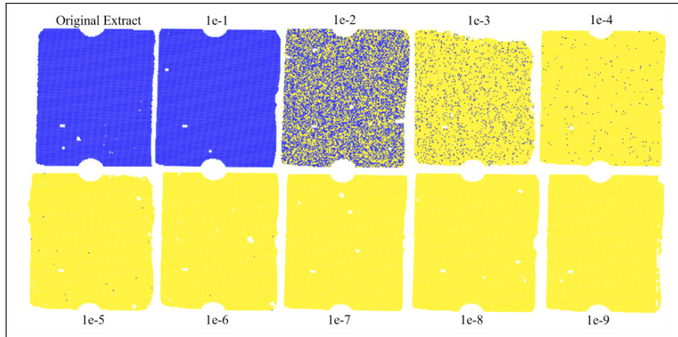

Fig. 2. Digital chip images for TaqMan assays to detect the16SrIV-A phytoplasma in a dilution series.

For the 16SrIV-D phytoplasma serial dilution, the original extract had a high enough titer to fully saturate the chip (Fig. 3). The $1e^{-1}$ dilution reaction displayed near complete saturation of the chip but did show some wells as negative (no target/no amplification) (Fig. 3). The $1e^{-3}$ dilution reaction was the optimal reaction (Fig. 3), with the pathogen detectable down to the $1e^{-6}$ dilution reaction (0.0001% diluted from original extract). At the $1e^{-7}$, $1e^{-8}$, and $1e^{-9}$ dilution reactions, the phytoplasma was undetectable (Fig. 3). For estimates of copies/µl, the $1e^{-2}$ gave the most accurate estimate (Table 3) while the $1e^{-1}$ reaction yielded an estimate with low accuracy (Table 3) due to the disproportionate number of positive wells to negative wells (Table 3). Estimates for copies/µl were also given for the $1e^{-3}$, $1e^{-4}$, $1e^{-5}$, and $1e^{-6}$ dilution reactions with decreasing accuracy from the $1e^{-2}$ reaction due to samples becoming too diluted for detection (Table 3). An estimate of copies/µl of the original extract from the reaction using original extract was not given due to a high titer, resulting in complete saturation of the chip (Table 3). The $1e^{-7}$, $1e^{-8}$, and $1e^{-9}$ dilution reactions also did not yield estimates of copies/µl for the original extract due to negative reactions across the entire chip (Table 3).

Table 3. Digital PCR data for the serial dilution of the 16SrIV-D isolate.

Dilution	Total Wells	(+) Wells	(-) Wells	Copies/µl	Precision
1	17,224	17,224	0	N/A	N/A
1e-1	17,306	17,074	232	5,905.10	3.13%
1e-2	17,979	8,378	9,601	833.55	2.21%
1e-3	18,401	1,131	17,270	84.03	6.00%
1e-4	16,797	129	16,668	10.21	18.84%
1e-5	17,435	29	17,406	2.21	43.90%
1e-6	15,214	3	15,211	8.71e-2	609.93%
1e-7	16,394	0	16,394	N/A	N/A
1e-8	17,529	0	17,529	N/A	N/A
1e-9	18,031	0	18,031	N/A	N/A
Healthy	18,222	0	18,222	N/A	N/A
Water	17,459	0	17,459	N/A	N/A

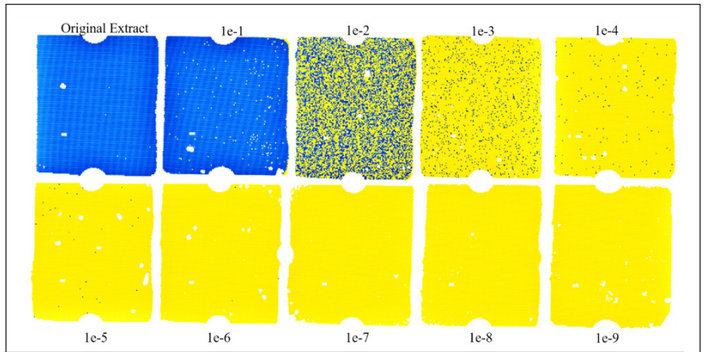

Fig. 3. Digital chip images for TaqMan assays to detect the16SrIV-D phytoplasma in a dilution series.

CONCLUSIONS & RECOMMENDATIONS

Based on these results, our detection capabilities have increased, allowing us to detect phytoplasmas at levels that were previously undetectable. These capabilities will allow us to detect phytoplasma earlier in the disease cycle and prior to symptoms development, in the salivary glands of vector candidates, and in pools of a larger number of samples.

This technology has been made available through our diagnostic clinic if sampling a palm with no symptoms, this is a good alternative and should be used in case the palm is at an earlier stage of infection where standard tools may not detect the phytoplasma.