Title:
Detection of Liberibacter asiaticus in a single infected Asian citrus psyllid adult or nymph: Impact of dilution with clean Asian citrus psyllids (Diaphorina citri) during extraction

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Abstract:
Now that the presence of Huanglongbing (HLB) has been confirmed in California, protecting the state's citrus industry through early detection of disease is essential in curtailing its spread. Because '*Candidatus* Liberibacter asiaticus' (Las), the putative causal agent of HLB, accumulates in its vector, the Asian citrus psyllid (ACP), *Diaphorina citri*, Kuwayama, accurate testing of

the insect is vital. Due to the fact that insect secondary metabolites interfere with downstream applications (1, 3) there is concern about the number of insects pooled in DNA extractions without compromising Las detection. The current USDA CPHST approved method of testing (2) limits the pooling size to five individual insects per extraction followed by the highly sensitive quantitative polymerase chain reaction (QPCR) detection technique. The QPCR reaction targets a region of the Las 16S ribosomal gene (4) and simultaneously the *D. citri* specific *wingless* (WGLS) gene (5) as an internal control for extraction efficiency. In contrast, the CDFA laboratory routinely pools up to 25 individual insects for DNA extraction, followed by the same QPCR detection. If pooling 25 individuals is indeed a safe practice all the labs currently limiting sample pooling to five individuals could save a substantial amount of time and money. We decided to approximate the pooling limit using single insect equivalents of DNA extracted from Las infected ACP from colonies at USDA-ARS in Fort Pierce, Florida pooled with intact ACP from clean UC Riverside, California, quarantine colonies. Depending on results we would follow these experiments with experiments using intact Las positive ACP.

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Detection of Liberibacter asiaticus in a single infected Asian citrus psyllid adult or nymph: Impact of dilution with clean Asian citrus psyllids (Diaphorina citri) during extraction

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Now that the presence of Huanglongbing (HLB) has been confirmed in California, protecting the state's citrus industry through early detection of disease is essential in curtailing its spread. Because '*Candidatus* Liberibacter asiaticus' (Las), the putative causal agent of HLB, accumulates in its vector, the Asian citrus psyllid (ACP), *Diaphorina citri*, Kuwayama, accurate testing of the insect is vital. Due to the fact that insect secondary metabolites interfere with downstream applications (1, 3) there is concern about the number of insects pooled in DNA extractions without compromising Las detection. The current USDA CPHST approved method of testing (2) limits the pooling size to five individual insects per extraction followed by the highly sensitive quantitative polymerase chain reaction (QPCR) detection technique. The QPCR reaction targets a region of the Las 16S ribosomal gene (4) and simultaneously the *D. citri* specific *wingless* (WGLS) gene (5) as an internal control for extraction efficiency. In contrast, the CDFA laboratory routinely pools up to 25 individual insects for DNA extraction, followed by the same QPCR detection. If pooling 25 individuals is indeed a safe practice all the labs currently limiting sample pooling to five individuals could save a substantial amount of time and money. We decided to approximate the pooling limit using single insect equivalents of DNA extracted from Las infected ACP from colonies at USDA-ARS in Fort Pierce, Florida pooled with intact ACP from clean UC Riverside, California, quarantine colonies. Depending on results we would follow these experiments with experiments using intact Las positive ACP.
Because the level of Las infection in an ACP colony is often under 30%, individual insect DNA extracts from the infected colonies were evaluated by QPCR to identify positives and the DNA pooled. DNA equivalent to a single positive psyllid was added to 4, 9, 14, 19 or 24 intact clean psyllids and extracted and the presence of Las determined by QPCR. While the CRB-CPDPP laboratory performed the single psyllid extractions and provided the pooled positive psyllid DNA

samples and clean ACP, the other five laboratories participated in the extraction and evaluation portion of the project. Each lab used either the USDA CPHST approved protocol employing the modified Qiagen DNeasy® Blood and Tissue kit or the modified Qiagen MagAttract® 96 DNA Plant Core Kit protocol, or both. In addition, the CDFA lab used an automated version of the DNeasy procedure, while the USDA-ARS Germplasm Repository lab used an automated version of the MagAttract procedure. In addition, the USDA-ARS Parlier lab used the traditional CTAB extraction method.

The pooling of *Candidatus* Liberibacter solanacearum (Lsol) infected and clean potato psyllids, *Bactericerca cockerelli*, from colonies at the USDA-ARS Repository in Riverside was studied as a control to verify the validity of using pooled positive DNA. Potato psyllids generally acquire Lsol at rates of 100%. Therefore, in addition to experiments using pooled potato psyllid DNA equivalent to a single Lsol positive psyllid, single intact potato psyllids from the Lsol positive colony were extracted with 4, 9, 14, 19 or 24 clean potato psyllids and the presence of Lsol determined by QPCR.

Both the C_Ts for Las (Figure 1.) as well as WGLS (Figure 2) obtained by all of the participating laboratories were essentially identical, regardless of the protocol used, whether manually or automated. Las was detected at roughly the same level in extractions where 24 clean ACP were extracted with a single insect equivalent of Las positive DNA as when 4 clean ACP were used for pooling and all values were within the positive range. There was some loss of recovery of the Las positive single insect equivalent when extracted alone. This was also seen across all labs and protocols. The cause of this loss is unclear since the extraction of intact single ACP seem to be efficient based on the Las and WGLS Cts obtained when screening for positives to pool.

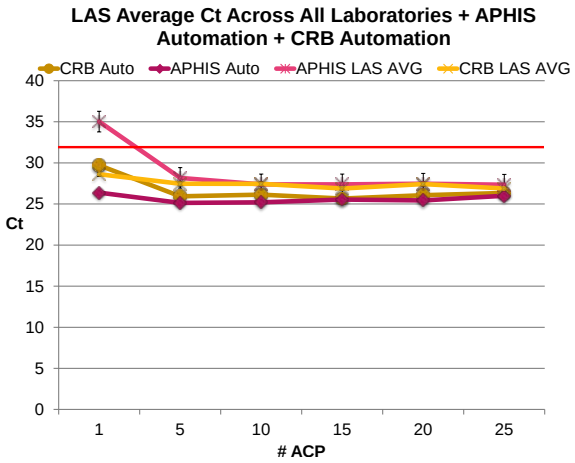


Figure 1. Las average Cts. APHIS = DNeasy protocol CRB = MagAttract protocol. Cts represent positives if below 32 (red line).

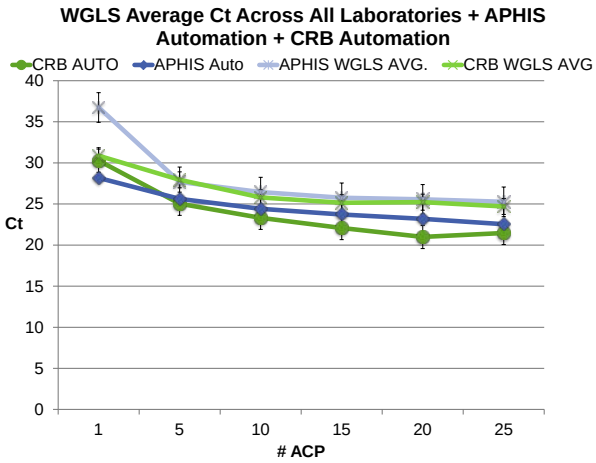


Figure 2. WGLS average Cts. APHIS = DNeasy protocol CRB = MagAttract protocol.

Experiments with Lsol positive potato psyllids provide evidence that the approach of using a single insect equivalent of pooled DNA does approximate that obtained with individual intact potato psyllids. There was, understandably, more variation in the data obtained with the intact psyllids since the extent of infection in individuals varies, but overall the Cts for Las and WGLS were very similar.

Since all the participating labs obtained similar results in the ACP study, the CRB-CPDPP lab extended the ACP study to pooling of the single insect equivalent of Las positive DNA with 49

and 99 clean ACP. Because there was no significant difference in the Cts obtained with the APHIS and CRB protocols in the joint study, the CRB protocol was used for these experiments. Varying amounts of the initial lysing buffer were used; the usual volume of 300 µl, then 600 µl and 800 µl.

All Cts obtained for Las were within the positive range for all extractions (Figure 5). Likewise WGLS Cts did not vary greatly with the exception of one point (Figure 6). Together these data indicate that the practice of pooling 25, or even more ACP, does not detrimentally affect Las detection.

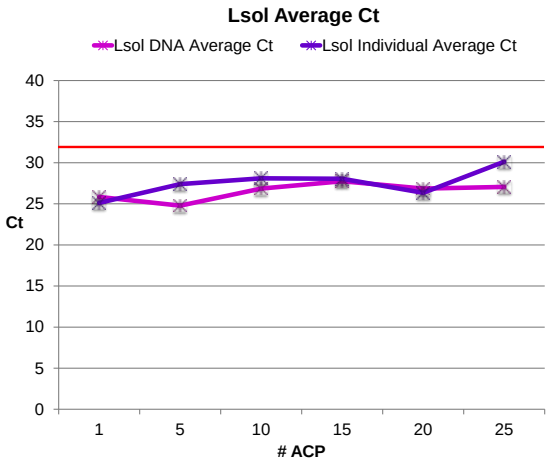


Figure 3. Lsol average Cts. APHIS = DNeasy protocol CRB = MagAttract protocol. Cts represent positives if below 32 (red line).

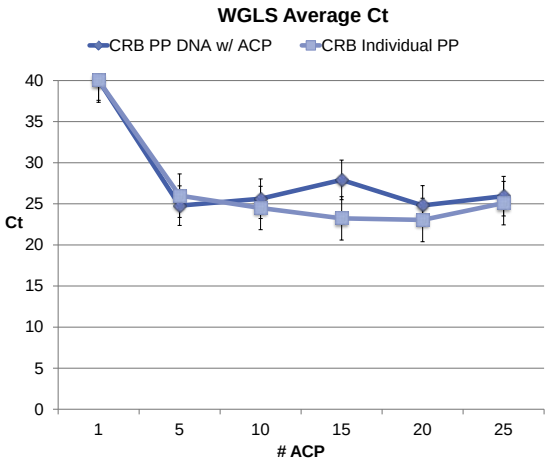


Figure 4. WGLS average Cts. APHIS = DNeasy protocol CRB = MagAttract protocol.

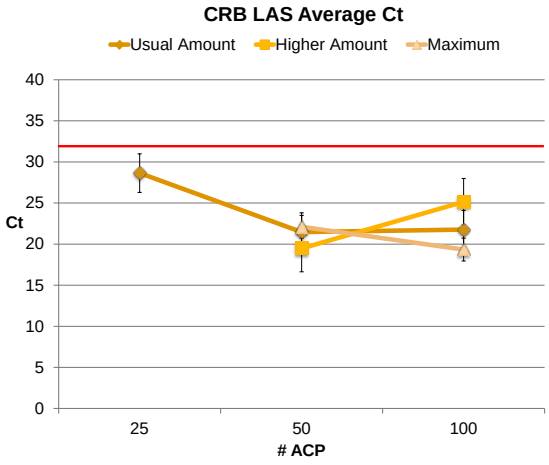


Figure 5. Las average Cts. Usual = 300 µl, Higher = 600 µl, Maximum = 800 µl. Cts represent positives if below 32 (red line).

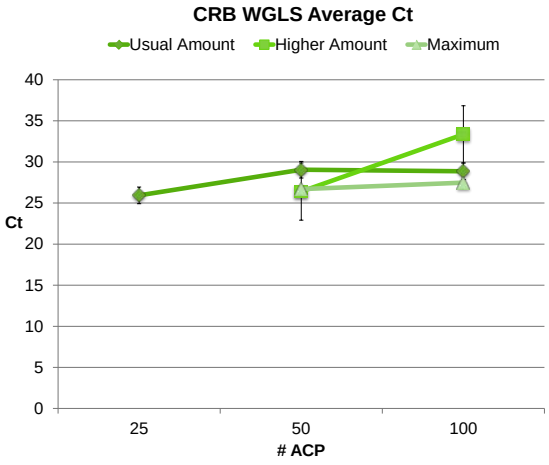
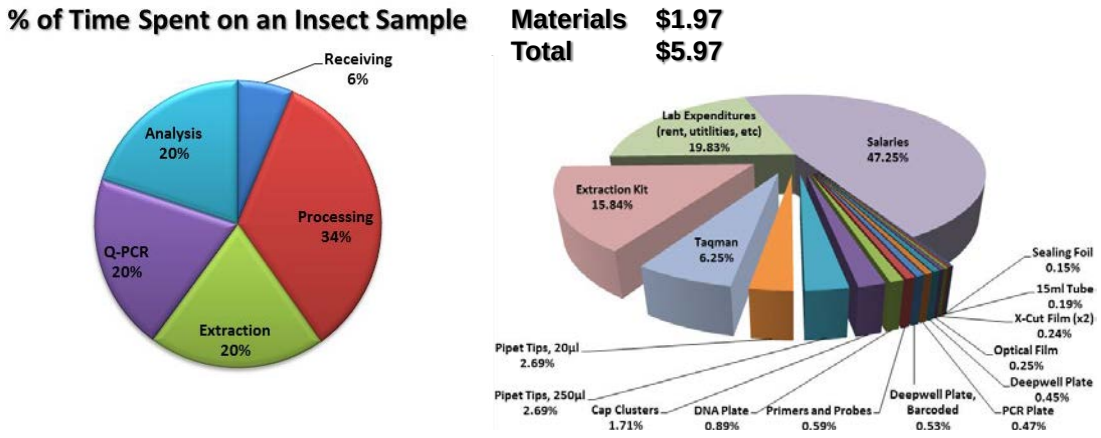


Figure 6. WGLS average Cts. Usual = 300 µl, Higher = 600 µl, Maximum = 800 µl.

These data also indicate that significant savings in both time and money could be made by pooling larger numbers of ACP. A cost analysis of the time and expense involved in processing each sample in the CRB-CPDPP lab is presented in Figure 7. Out of 33,756 samples processed 11,035 contained five or fewer ACP and would have been processed in a single well regardless. However, 22,721 samples represented subsamples of samples having greater than five ACP.

Assuming that all subsamples contained five insects, if samples were pooled into subsamples of 25 ACP, only an additional 4,544 samples would have needed to be extracted and analyzed. This represents a 54% reduction in the number of samples. At $1.97 per sample, a savings of $35,808 could have been obtained on the reagents alone. There also would be a significant reduction in the time involved in sampling.

COST ANALYSIS FOR ACP PROCESSING @ 5 ACP PER WELL



Since these experiments were done using a single insect equivalent of Las positive ACP DNA rather than intact insects, the participating laboratories plan to repeat these studies using enough single ACP at each pooling level to obtain statistically significant results.

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