Methods for Rapid and Effective PCR-Based Detection of ‘Candidatus Liberibacter solanacearum’ from the Insect Vector Bactericera cockerelli: Streamlining the DNA Extraction/Purification Process

Author(s): Julien Lévy, Joseph Hancock, Aravind Ravindran, Dennis Gross, Cecilia Tamborindegy, and Elizabeth Pierson


Published By: Entomological Society of America

URL: http://www.bioone.org/doi/full/10.1603/EC12419
ABSTRACT This study provides a protocol for rapid DNA isolation from psyllid vectors (Bactericera cockerelli and Diaphorina citri) that can be used directly with DNA-based methods for the detection of ‘Candidatus (Ca.) Liberibacter solanacearum,’ the bacterial causal agent of potato zebra chip disease and eventually for ‘Ca. Liberibacter asiaticus’ the causal agent of huanglongbing disease in citrus. The fast DNA extraction protocol was designed to work with conventional polymerase chain reaction (cPCR) DNA amplification as well as Loop mediated PCR DNA amplification. Direct cPCR of the psyllid 28S rDNA gene from samples prepared using the fast DNA extraction method was as reliable as from samples prepared using standard DNA purification (>97% from live insects) as tested in B. cockerelli. However, samples prepared using the fast DNA extraction method had to be diluted 1:100 in sterile water for reliable amplification, presumably to dilute PCR inhibitors in the crude extract. Similarly, both cPCR and loop mediated PCR DNA amplification detected ‘Ca. Liberibacter’ in psyllids infected with either the zebra chip or huanglongbing pathogen equally well from diluted samples prepared using the fast DNA extraction method or from samples prepared using a DNA purification step. In addition to being reliable, the time required to complete the fast DNA extraction for 10 samples was on average ~5 min and required no special reagents or laboratory equipment. Thus, the fast DNA extraction method shows strong promise as a rapid, reliable, and expedient method when coupled with PCR-based analyses for detection of ‘Ca. Liberibacter’ pathogens in psyllids.

KEY WORDS pathogen detection, Bactericera cockerelli, Zebra chip, Candidatus Liberibacter solanacearum, LAMP

Zebra chip (ZC) disease of potato is caused by the nonculturable, phloem-limited, gram-negative bacterium identified as ‘Candidatus (Ca.) Liberibacter solanacearum’ (Lso). This pathogen is vectored to solanaceous hosts by the potato/tomato psyllid (PTP) Bactericera cockerelli (Hemiptera: Triozidae) (Munyaneza et al. 2007, Hansen et al. 2008, Liefting et al. 2009a, Secor et al. 2009). The earliest disease symptoms include leaf curling and chlorosis, and shortened internodes; as the disease progresses symptoms include purple leaves, swollen internodes, aerial tubers, and ultimately wilting and premature death (Munyaneza et al. 2007). The trademark of the disease is the appearance of dark ‘stripes’ along the medullary rays in the potato tubers, which become more pronounced when tubers are fried, making them unsuitable for potato chip or French fry production (Rivera–Varas 2004). ZC was first reported in Mexico in 1994 and subsequently in south Texas in 2000 (Rivera–Varas 2004). Since then, it has spread to other western potato producing states in the United States including: Arizona, California, Colorado, Idaho, Kansas, Nebraska, Nevada, New Mexico, Oregon, Wyoming, and Washington (Crosslin et al. 2010, 2011b). ZC also is a threat in other potato producing countries including Mexico, Guatemala, and Hondur as in the Americas and New Zealand (Rivera–Varas 2004, Liefting et al. 2008, Munyaneza et al. 2009). In addition to potato, Lso has been found in other economically important solanaceous hosts including tomato, pepper, eggplant, tamarillo, and cape gooseberry as well as uncultivated solanaceous hosts including wolfberry (Lycium berlandieri), silverleaf nightshade (Solanum elaeagnifolium), and buffalobur nightshade (S. rostratum), which potentially serve as reservoirs for the pathogen and vector (Liefting et al. 2009b). A closely related ‘Ca. Liberibacter’ species, ‘Ca. Liberibacter asiaticus’ (Las) is the causative agent of huanglongbing (HLB) disease in citrus (also referred to as citrus greening) in the United States (Bové 2006). Similar to Lso, Las
is transmitted by a psyllid vector, the Asian citrus psyllid (ACP) *Diaphorina citri* (Hemiptera: Psyllidae) (Capoor et al. 1967, Chen et al. 1973). Although plant diseases caused by psyllid transmission of ‘Ca. Liberibacter’ were not observed in the United States before 2000, they are now threatening both potato and citrus production and have the potential for causing economic damage on other commercial crops.

Currently, there are no effective control strategies for protecting plants from ‘Ca. Liberibacter’ species and existing management practices focus on accurate and timely detection of psyllids vectoring the pathogen and effective insect control to limit the spread of infection among field plants. Given the swift geographic expansion of ‘Ca. Liberibacter’-caused diseases, there is a considerable demand for rapid and effective high-throughput methods for detection of ‘Ca. Liberibacter’ in insect and plant samples. Current methods for detecting ‘Ca. Liberibacter’ species in insects and plants rely primarily on conventional PCR (cPCR) assays, although methods using quantitative real-time PCR (qPCR) also have been developed (Li et al. 2009, Levy et al. 2011). Protocols for cPCR are based on the use of primers designed for the 16S rDNA sequence, 16–23S rDNA intergenic region, or the housekeeping gene adenylate kinase ( *adk*) of different ‘Ca. Liberibacter’ species (Hansen et al. 2008, Li et al. 2009, Crosslin et al. 2011a, Ravindran et al. 2011). Despite progress in optimizing detection methods for increased reliability (Ravindran et al. 2011), they remain time consuming (*e.g.*, require pre-PCR DNA purification and post-PCR analysis via gel electrophoresis or qPCR software). A relatively new technology for bacterial pathogen detection known as loop-mediated isothermal amplification (LAMP) was developed recently for both Lso and Las detection (Ravindran et al. 2012), with the goal of streamlining the detection pipeline. LAMP is a PCR method based on autocycling strand displacement facilitated by *Bacillus stearothermophilus* (Bst) DNA polymerase (Tomita et al. 2008). A by-product of the LAMP amplification is the formation of an insoluble white precipitate of magnesium pyrophosphate, which becomes increasingly visible with time in the reaction tube. LAMP has the advantages of being as reliable as cPCR for Lso and Las detection, but faster in that post-PCR analysis is not required (Ravindran et al. 2012).

In this study, we present methods designed to further streamline the detection process by reducing the amount of time required for DNA preparation before PCR-based amplification. Although methods for DNA extraction and purification vary among research groups, this step is typically the most time and effort consuming. However, DNA extraction and purification has been considered crucial for reliable diagnostics of bacteria in insect samples because of the need to remove potential PCR inhibitors, especially phenolic compounds present in insect cuticles that may be released when insects are homogenized. Previous research demonstrated that insect DNA can be successfully amplified from crude DNA extracts (Gloor and Engels 1992, Huang et al. 2009). This article describes methods for rapid DNA isolation from insect vectors that can be used with traditional PCR analyses or in conjunction with the stream-lined LAMP method for rapid and robust detection of bacterial symbionts including plant pathogens. We demonstrate that adaptation of these direct PCR methods result in significant savings in time and labor, without decreasing reliability of detection. Although we focus on diagnostics for Lso in *B. cockerelli*, we show that the method also has potential for the detection of Las from *D. citri* samples.

### Materials and Methods

**Insect Source.** *B. cockerelli* from Lso-infected and Lso-uninfected psyllid colonies described previously (Nachappa et al. 2011) were maintained in growth chambers in our laboratory at Texas A&M University in College Station. The insect colonies were maintained on potato or tomato plants in separate 14” by 14” by 24” insect cages (BioQuip, Rancho Dominguez, CA) at a constant temperature of 22°C and photoperiod of 14:10 (L:D) h. Dead insects from sticky traps were obtained either from traps placed within our insect growth rooms or from potato fields located in Weslaco, TX, during February and March 2012. *D. citri* from Las-infected colonies were shipped frozen on dry ice by Dr. T.A. Ebert at the University of Florida Citrus Research and Education Center in Lake Alfred, FL.

**Fast DNA Extraction Method.** Samples included either individual insects or multiple insect samples (three or five insects per sample). The fast DNA extraction method was modified from a previously reported protocol developed for direct PCR amplification from *Drosophila* DNA extractions (Gloor and Engels 1992). Briefly, independent samples of live insects (nymphs or adults, single or multiple insect samples) from our laboratory colonies or dead insects obtained from yellow sticky traps (BioQuip) were placed into separate 1.5-ml microcentrifuge tubes. Insects were directly homogenized with either a sterile micropestle (VWR) or a sterile disposable micropipette tip (VWR). Homogenized material was resuspended in a total volume of 75 μl of sterile water by pipetting. Samples typically were used directly, but occasionally stored briefly at −20°C. Unless otherwise stated, samples were diluted 1/100 for PCR and LAMP analyses.

**DNA Extraction and Purification Method.** The standard method used in our laboratory for the extraction and purification of DNA from psyllids uses CTAB (cetyltrimethylammonium bromide) in the extraction buffer and is modified from the extraction protocol previously published by (Reineke et al. 1998). Briefly, each psyllid sample was ground to a fine powder in liquid nitrogen using a sterile micropestle. Five hundred microliters of extraction buffer (0.1M Tris, 10 mM EDTA, 2% SDS, and pH 8.0) were added to the sample and ground again with a pestle. The sample was then incubated for 1 h at 55–60°C, and afterwards 140
μl of 5M NaCl and 65 μl CTAB were added. After, 10 min incubation at 65°C, 700 μl of a mixture of chloroform: isoamyl alcohol (24:1 ratio) were added to the sample. The sample was centrifuged at 13,000 x g for 10 min at 4°C and the supernatant transferred to a new tube, where 250 μl of 5 M ammonium acetate were added. The sample was incubated on ice for 30 min and then centrifuged at 13,000 x g for 10 min at 4°C. The supernatant was then discarded, and the pellet was first washed in cold isopropanol and then 75% ethanol. After each wash, the sample was centrifuged at 13,000 x g for 10 min at 4°C and the supernatant discarded. The remaining pellet was then dried in a vacuum centrifuge and resuspended in 70 μl sterile water.

**Conventional PCR.** The PCR reactions (15 μl final volume) were conducted using the GoTaq Master Mix Promega Corp. (Madison, WI) and the following volumes of reagents: 1 μl of DNA extract, 7.5 μl of Master Mix, 1 μl of each primer (10 mM), and 4.5 μl sterile water. The PCR amplification was performed using an Eppendorf Thermocycler (Hamburg, Germany) using the following reaction conditions: (95°C for 3 min; 40 cycles of 95°C for 40 s, 55°C for 40 s, and 72°C for 1 min; and 72°C for 10 min. PCR products (all 15 μl) were resolved by electrophoresis in 1% agarose gels stained with ethidium bromide. As described for the conventional PCR amplification of the Lso or Las target was determined previously (Ravindran et al. 2012). LAMP PCR reactions (15 μl final volume) were conducted using 1 μl of either undiluted extract (1/1) or extract diluted 1/10 or 1/100 in sterile water. The 1/1 and 1/10 dilutions failed to amplify (no band) the psyllid target gene (0% success), but the 1/100 dilution was highly effective (100% success). At the start, we used pools of several insects (three or five insects per sample) to ensure that we obtained sufficient DNA for the amplification of the psyllid 28S rDNA gene. The PCR amplification was conducted using 1 μl of either undiluted extract (1/1) or extract diluted 1/10 or 1/100 in sterile water. The 1/1 and 1/10 dilutions failed to amplify (no band) the psyllid target gene (0% success), but the 1/100 dilution was highly effective (100% success) (Table 1). We also conducted tests using the fast DNA extraction protocol on samples obtained from single insects. Again, the PCR amplification was conducted using 1 μl of either undiluted extract (1/1) or extract diluted 1/10 or 1/100 in sterile water. Similar to the multiple insect samples, PCR amplifications were reliable only when the samples were diluted 1/100 (100% success), despite the smaller amount of starting material. In contrast to the multiple insect samples, some amplification was achieved for the undiluted and 1/10 diluted samples (∼10 and 50%, respectively).

**Results and Discussion.**

**Optimization of Methods.** The first goal was to optimize the method for extracting DNA from single or multiple insects. Several methods were tested for immobilizing the insects and for inexpensively grinding insects in a disposable 1.5 ml Eppendorf tube. The immobilization methods tested included no immobilization or immobilization by either flash freezing in liquid nitrogen or by placing insects on ice for 10 min before grinding. Grinding methods included use of a sterile micropipette or a sterile disposable micropipette tip (a less expensive option). For single insects, immobilization was not required and both grinding implements (micropipette or disposable micropipette tip) were sufficient. For multiple insects, immobilization by either technique was required and both were equally effective; grinding with the micropipette was the preferred method for adequate homogenization of insects. Initially the fast protocol included two additional steps: an incubation step (15 min at 65°C) after grinding and a 5 s spin in a table-top centrifuge to pellet the insect debris. Subsequently, in an effort to reduce time, equipment, and labor, both the incubation at 65°C and the centrifugation step were removed from the protocol with no change in efficacy (data not shown). Thus, the method with no incubation or centrifugation steps is recommended and was used for all experiments.

**Importance of Diluting the Extract.** Given the possibility that the fast DNA extraction protocol would not remove potential Taq inhibitors from single, and especially multiple insect samples, we tested the importance of diluting the DNA extract for reliable PCR amplification. At the start, we used pools of several insects (three or five insects per sample) to ensure that we obtained sufficient DNA for the amplification of the psyllid 28S rDNA gene. The PCR amplification was conducted using 1 μl of either undiluted extract (1/1) or extract diluted 1/10 or 1/100 in sterile water. The 1/1 and 1/10 dilutions failed to amplify (no band) the psyllid target gene (0% success), but the 1/100 dilution was highly effective (100% success) (Table 1). We also conducted tests using the fast DNA extraction protocol on samples obtained from single insects. Again, the PCR amplification was conducted using 1 μl of either undiluted extract (1/1) or extract diluted 1/10 or 1/100 in sterile water. Similar to the multiple insect samples, PCR amplifications were reliable only when the samples were diluted 1/100 (100% success), despite the smaller amount of starting material. In contrast to the multiple insect samples, some amplification was achieved for the undiluted and 1/10 diluted samples (∼10 and 50%, respectively).

Given the importance of dilution for reliable PCR amplification, but the significantly smaller amount of bacterial DNA present in each DNA extraction from insects, we also examined how sample dilution would affect amplification of bacterial DNA from the insect samples. Although the percentage of individuals in our colonies infected with Lso varies over time, ∼30–50% of the insects in our laboratory population were infected when insects were collected for this assay (de-


**Table 1.** Importance of dilution for the reliable amplification of insect and bacterial DNA from multiple insect (3–5 insects per sample) or single insect samples extracted using the fast DNA extraction method

<table>
<thead>
<tr>
<th>Dilution (extract/water)</th>
<th>Multiple insect samples</th>
<th>Single insect samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Insect (28S rDNA gene)</td>
<td>Lso (16S-23S rDNA gene)</td>
</tr>
<tr>
<td></td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>1×</td>
<td>3/25</td>
<td>2/25</td>
</tr>
<tr>
<td>1/10</td>
<td>3/25</td>
<td>2/25</td>
</tr>
<tr>
<td>1/100</td>
<td>25/25</td>
<td>8/25</td>
</tr>
</tbody>
</table>

Homogenized insect material was suspended in a total vol of 75 µl of sterile water. The PCR amplification was conducted using 1 µl of either undiluted extract (1×) or extract diluted 1/10 and 1/100 in sterile water. For the detection of insect vs bacteria DNA, the insect 28S rDNA gene and the intergenic region of the bacterial 16S and 23S rDNA genes were used as the PCR targets, respectively.

determined from routine population sampling). Using the fast DNA extraction method, the Lso target amplified only in the 1/100 diluted samples from the multiple insect DNA extracts (100% of samples) and most frequently in the 1/10 diluted samples from the single insect DNA extracts (32% as compared with 8% in less diluted samples) (Table 1). Detecting Lso in every multiple insect sample is not surprising given that approximately one-third of the single insect samples were positive for Lso and three or five insects were used in the multiple insect samples.

**Efficacy of the Fast Method on Different Insect Life Stages or on Dead (Trapped) Insects.** To assess the efficiency of this new protocol on different types of insect samples, the fast method was used to extract DNA from live adults taken from field and laboratory populations, fifth instar nymphs (from laboratory populations), and dead insects taken from sticky traps (used to collect insects from the laboratory and field). PCR amplification of the insect 28S rDNA target was successful from ≈94% of all fast DNA extraction samples (≈98% of live adult, 88% of nymph, and 86% of dead insect samples) (Table 2). Overall these results are similar to the rates we routinely obtain using our standard DNA purification method (97% for live adults, n = 140). The lower amplification rate from dead insects (86%) is a function of some of the insects on sticky cards being extremely degraded and/or covered in glue, and in this degraded state occasionally being misidentified as psyllids.

**Efficacy of the Fast Method for the Detection of Lso in Insect Samples.** The next step was to determine whether the fast DNA extraction protocol coupled with conventional PCR (e.g., direct PCR) was as reliable as conventional PCR following a DNA purification step (e.g., the current standard protocol). Because DNA cannot be extracted from the same insect using both protocols and the frequency of individuals infected with Lso can vary temporally, all insects were collected for method comparison at the same time (on a sticky trap placed in the insect rearing room) and randomly assigned to a method. Both extraction methods resulted in PCR amplification of the insect target 28S rDNA (positive control) from all insects sampled and similar infection rates were observed using both DNA extraction methods (Table 3). The extraction methods also were compared using samples of insects obtained from a sticky trap placed in a potato field in Weslaco, TX. Both extraction methods resulted in PCR amplification of the insect target 28S rDNA from all 10 insects sampled. The frequency of field insects infected with Lso was 1/10 using both DNA extraction methods (data not shown), which corresponds with the findings from larger field samples taken at that time (5–10%) (Henne 2012).

**Fast Method Can be Successfully Coupled With LAMP Detection of Lso.** Given the possibility that the fast DNA extraction protocol would not remove potential Bst inhibitors, it was unclear whether the BstDNA polymerase used in the LAMP amplification would reliably amplify bacterial targets from single insect samples prepared using the fast DNA extraction method. For this analysis 28 insects from the Lso-infected laboratory colony, 17 insects from the Lso-uninfected laboratory colony, and 3 insects from field sticky traps were sampled. For each insect, DNA was extracted using the fast DNA extraction method and each sample (diluted 1/100 in sterile water) was tested for the presence of Lso using both conventional PCR and LAMP. For all insect samples prepared using the fast DNA extraction method, cPCR- method amplified the 28S rDNA insect target gene (positive control); therefore, those samples were suitable for testing by LAMP. The results for the amplification of the 16S rDNA bacterial target for the two PCR-based detection methods (cPCR and LAMP) are similar (Table 3, gray) although LAMP was slightly more sensitive than conventional PCR. The higher sensitivity of LAMP was not correlated with a higher false positive detection rate as all samples from the Lso-uninfected colony tested negative for Lso using both PCR-based methods. Slightly higher sensitivity of detection for Lso using LAMP was reported previously for comparisons between conventional PCR and LAMP following standard DNA purification methods (Ravindran et al. 2011).
For some of the insect samples prepared using the fast DNA extraction method the resulting DNA extract was analyzed using both conventional PCR (cPCR) and the LAMP PCR-based amplification method (LAMP-PCR). The percentage of samples in which Lso was detected is given parenthetically. Conditions that were not tested are labeled as not done (nd). Statistical analysis comparing the PCR amplification of samples prepared using either a DNA purification protocol or the fast DNA extraction method using the \( \chi^2 \) test show that there were no differences between the treatments (\( \chi^2 = 0; df = 1; P \text{ value} = 1 \)).

### Table 3. Efficacy of PCR amplification of the Lso 16S rDNA target from single insect samples prepared using either a DNA purification protocol or the fast DNA extraction method (with a 1/100 dilution of fast product in sterile water)

<table>
<thead>
<tr>
<th>Insect sample</th>
<th>DNA purification and cPCR</th>
<th>Fast extraction and cPCR</th>
<th>Fast extraction and LAMP-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults (laboratory sticky trap)</td>
<td>6/15 (40%)</td>
<td>5/14 (36%)</td>
<td>nd</td>
</tr>
<tr>
<td>Adults (infected colony)</td>
<td>nd</td>
<td>17/25 (61%)</td>
<td>19/25 (68%)</td>
</tr>
<tr>
<td>Adults (uninfected colony)</td>
<td>nd</td>
<td>0/17</td>
<td>0/17</td>
</tr>
<tr>
<td>Adults (field sticky trap)</td>
<td>nd</td>
<td>1/3</td>
<td>1/3</td>
</tr>
</tbody>
</table>

In conclusion, we describe a method showing promise for rapid DNA isolation from insect vectors that can be used with traditional PCR analyses or coupled with LAMP for rapid and reliable detection of bacterial symbionts including plant pathogens. As anticipated, the fast DNA extraction protocol apparently is not effective in removing PCR inhibitors from the crude extractions. Thus direct PCR amplification from single or multiple insect samples prepared using the fast DNA extraction protocol must be diluted 1/100 in sterile water for reliable cPCR or LAMP amplification. However, when diluted 1/100, direct cPCR amplification of the psyllid 28S rRNA sequence from samples prepared using the fast DNA extraction method was as reliable as cPCR amplification after DNA purification (98 and 97% success rate, respectively). Similarly LAMP amplification of the psyllid 28S rDNA sequence was highly effective (100% success rate) for samples prepared using the fast DNA extraction method indicating this method is robust enough for PCR amplification using either Taq or Bst DNA polymerase. Although the same insect samples could not be compared using both DNA sample preparation methods, cPCR detection of Lso also appeared to be equally effective when samples were prepared using the fast DNA extraction method or a DNA purification step (e.g., Lso was present in 36 and 40% of insects sampled, respectively). As demonstrated previously (Ravin-dran et al. 2012) the LAMP method was slightly more sensitive than cPCR in detecting Lso, even when samples were prepared using the fast DNA extraction protocol as in this study (61 vs. 68%, respectively).

In addition to being robust and reliable, the fast DNA extraction method resulted in significant savings in time, equipment, and labor. Using the optimized protocol (e.g., without the 15 min incubation [65°C] and subsequent centrifugation steps), the time required to complete the fast DNA extraction for 10 samples was 5 min. The laboratory equipment was minimal: only sterile water, a disposa ble 1.5 ml Eppendorf tube and sterile micropette, a laboratory pipettor, and sterile tips were required. Our standard CTAB DNA extraction and DNA purification protocol for 10 samples required over 2 h including multiple incubations (60, 10, and 30 min), multiple reagents (and access to reagent disposal capabilities), and multiple washing and centrifugation steps. Required equipment for the DNA purification included a table-top centrifuge, water bath, and vacuum centrifuge as well as several sterile plastic disposables per sample (tubes and tips). In contrast, the fast DNA extraction coupled with LAMP amplification can (and has—this study) been performed on the bed of a pick-up truck in <90 min. Further, the method was reliable in detecting the pathogen in samples composed of multiple
insects (3–5), indicating its reliability for pathogen detection in small batch samples, which would further expedite the process of detecting Lso or Las in insect populations. Thus, the fast DNA extraction method shows strong promise as a reliable, rapid, and cost-effective method for detection of ‘Ca. Liberibacter’ pathogens and potentially other microbial symbionts in psyllids and other related vectors.

Acknowledgments

Funding was provided by the Texas Department of Agriculture-Specialty Crop Research and Product Development Grant Program award SCFB-1213-014. We thank TA Ebert and Niam Wang for supplying field collected B. cockerelli, and O. Huot, S. Kay, and K. Davidson for independently testing the method in lab.

References Cited


Received 2 October 2012, accepted 14 February 2013.