A SYBR Green-Based Real-Time Polymerase Chain Reaction Protocol and Novel DNA Extraction Technique to Detect *Xylella fastidiosa* in *Homalodisca coagulata*

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ABSTRACT *Homalodisca coagulata* Say (Hemiptera: Cicadellidae) is a major agronomic pest because it transmits *Xylella fastidiosa* (Wells), the bacterium that causes Pierce's disease of grapevine. The ability to easily detect *X. fastidiosa* in populations of *H. coagulata* facilitates epidemiological studies and development of a monitoring program supporting disease management. Such a program depends on a detection protocol that is rapid, reproducible, and amenable to large sample sizes, while remaining sensitive enough to detect low amounts of pathogen DNA. In this study, we developed an improved method to speed DNA extraction by implementing a simple vacuum step that replaces labor- and time-intensive maceration of tissue samples and that is compatible with manufactured DNA extraction kits. Additionally, we have developed a SYBR Green-based real-time (RT)-polymerase chain reaction (PCR) system, which uses traditional PCR primers that are relatively inexpensive and effective. Using this extraction/RT-PCR system, we found no statistically significant differences in the detection of *X. fastidiosa* among samples that were either immediately extracted or stored dry or in mineral oil for 10 d at −4°C. In further testing, we found no significant reduction in detection capabilities for *X. fastidiosa*-fed *H. coagulata* left in the sun on yellow sticky cards for up to 6 d. Therefore, we recommend a field-based detection system that includes recovery of *H. coagulata* from sticky traps for up to 6 d after trapping, subsequent freezing of samples for as long as 10 d before vacuum extraction is performed, and detection of the bacterium by SYBR Green-based RT-PCR.

GRAPEVINES INFECTED WITH *Xylella fastidiosa* (Wells), the bacterium that induces Pierce's disease of grapevine (Hopkins 1977), usually die within 3 to 5 yr after infection due to the occlusion of xylem vessels (Purcell 1997). The glassy-winged sharpshooter, *Homalodisca coagulata* Say (Hemiptera: Cicadellidae) recently became an important vector of *X. fastidiosa* in California, spreading *X. fastidiosa* to grapevines that traditionally had little or no Pierce's disease (Purcell 1997, Almeida and Purcell 2003a). This vector can disperse widely (Blua and Morgan 2003) and has a large host range (Redak et al. 2003), resulting in alarming spread of *X. fastidiosa* to new areas (Hoddle et al. 2003). The presence of *H. coagulata* in new regions of California, greater incidences of *X. fastidiosa*-induced diseases in several crops, including grapevine (Perring et al. 2001), almond (Almeida and Purcell 2003b), and oleander (Costa et al. 2000), and the threat of citrus variegated chlorosis (not currently found in the United States), has led to great concern over the ecology of this pest/pathogen interaction.

Over the past several years control programs have focused on reducing pathogen spread by managing vector populations (Redak et al. 2003). Improvements of these strategies can be achieved through studies examining patterns of disease epidemiology (Smart et al. 1998, Perring et al. 2001), and *H. coagulata* population densities and dispersion (Sorensen and Gill 1996, Blua and Morgan 2003, Hoddle et al. 2003). Most epidemiological studies of this system have involved *X. fastidiosa*'s interaction with host plants (Brlansky et al. 1991, Smart et al. 1998, Perring et al. 2001, Bextine and Miller 2004) or the population and behavioral ecology of the pest insect (Blua and Morgan 2003, Hoddle et al. 2003). Investigations of the interactions between *X. fastidiosa* and insect vectors have largely been limited to laboratory and greenhouse studies (Costa et al. 2000, Almeida and Purcell 2003a, Bextine et al. 2004). Molecular protocols, such as polymerase chain reaction (PCR), to detect *X. fastidiosa* in plants, have been developed and are currently being used in epidemiological studies in other disease systems (Sherald and Lei 1991; Minsavage et al. 1993; Pooler and Hartung 1995; Smart et al. 1998; Chen et al. 1999, 2000). Unfortunately, methods adapted to detect *X. fastidiosa* in insects are inefficient. We recently developed a DNA extraction protocol by using the DNeasy tissue extraction kit (QIAGEN, Valencia, CA) to detect *X. fastidiosa* in infectious *H. coagulata* (Bextine et al. 2004). Using this protocol, we reliably detected 50–500 *X. fastidiosa* cells with *H. coagulata* background using PCR-based detection.

A detection method designed for epidemiological studies, from collection of insect specimens to analysis

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of plant and animal samples for the presence of *X. fastidiosa*, needs to be rapid, reproducible, inexpensive, and amenable to large sample sizes. The current method for such an endeavor involves labor-intensive maceration of tissue samples (Bextine et al. 2004) and time-consuming agrose gel preparation for visualization of PCR products. In this study, we have developed a novel method for removing *X. fastidiosa* cells from the foregut and mouthparts of *H. coagulata*, where the bacterium resides in infectious insects (Brlansky et al. 1983). This method reduces maceration of nontarget insect tissue which, in turn, reduces the probability that PCR inhibitors will interfere with detection of the pathogen in the detection process (Bextine et al. 2004). In addition, we developed a quantitative real-time PCR (QRT-PCR) protocol to detect *X. fastidiosa* based on SYBR Green technology (Bates et al. 2001), which is cost-effective and rapid. In addition, we developed a reliable insect collection protocol that includes trapping and storage of samples before detection.

Materials and Methods

Collection and Maintenance of *H. coagulata*. *H. coagulata* adults were collected with a net from orange, *Citrus sinensis* (L.), trees at the University of California, Riverside. Test insects were transported to the laboratory in 30 by 30 by 30-cm collapsible, chiffon-screened field collecting cages (Bioquip Products Inc., Gardena, CA) and maintained in an environmentally controlled rearing room on chrysanthemum, *Chrysanthemum grandiflora* (L.) 'White Diamond', plants (photoperiod of 12:12 [L:D] h at 27°C and 70% RH).

***X. fastidiosa* Acquisition by *H. coagulata*.** Initially, 200 adult *H. coagulata* were placed in rearing cages containing 30–45-cm cuttings of *X. fastidiosa*-infected grapevines that showed Pierce's disease symptoms (Hopkins 1977) and that had previously tested positive by *X. fastidiosa*-specific enzyme-linked immunosorbent assay. We subsequently determined that a ratio of ten 30–45-cm infected grapevine cuttings per 100 *H. coagulata* was sufficient to sustain the insects. Cuttings were prepared by removing ≈20 cm of the leaves from the cut end of the branch and trimming an additional 3 cm of stem while submerging it in an aqueous solution of Floralife Liquid Flower Food (Floralife Inc., Walterboro, SC), prepared according to label instructions. The freshly trimmed cuttings were evenly spaced in a 0.95 L mason jar with Floralife solution. To prevent *H. coagulata* from drowning during the acquisition period, the mouth of the jar was covered with Parafilm (Pechiney, Plastic Packaging, Menasha, WI), before piercing the cut ends through the Parafilm into the container. Two jars each with 10 grapevine cuttings were placed in a ventilated, 60 by 30 by 60-cm rearing cage to which were added 10 *H. coagulata* per cutting. The cage was covered immediately with a lightweight dark cloth for 24 h. We have observed that this "calming down" period reduces mortality due to postcapture hyperactivity. The cloth

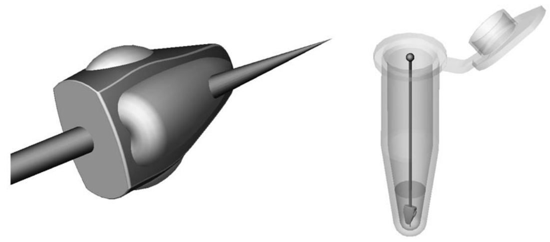

Fig. 1. Pinning of *H. coagulata* head. (A) View shows the angle of the pin coming through the back of a severed head and protruding through the frons and missing the eyes. (B) Pinned head is placed in a 1.5-μl microcentrifuge tube and submerged in a lytic buffer.

was removed after 24 h and *H. coagulata* were allowed to continue feeding on the *X. fastidiosa*-symptomatic vines for another 2 d before fresh cuttings were added to the cage. *H. coagulata* were allowed to feed for an additional 3 d, for a total acquisition access period of 6 d.

DNA Extraction. After the 6-d acquisition access period, all of the *H. coagulata* were cooled and beheaded with a sterile razor blade by cutting from the suture anterior to the forelegs to the suture posterior to the pronotum. Because the insect's head floats, an insect pin (size 3) (Bioquip Inc., Rancho Dominguez, CA) was placed through the back of the insect head and forced through the frons, so that the tip of the pin protruded slightly (Fig. 1A). When placing the pin, care was taken not to puncture the eyes, a potential source of PCR inhibitors (Bextine et al. 2004). The pinned head was then placed in a microcentrifuge tube (one per tube), and 500 μl of phosphate-buffered saline (PBS) was added to the tube so that the head was completely submerged (Fig. 1B) (note, a 96-well microtiter plate could be used in place of microcentrifuge tubes). All microcentrifuge tubes were loaded into a tube rack and placed in a glass vacuum desiccator (Fig. 2). The desiccator lid was put in place. To keep the buffer from being displaced from its tube, a vacuum was applied slowly until it reached 20 bars and was held for 15 s. Then, the slow release valve was opened, and pressure was slowly returned to ambient after ≈30 s. The vacuum application and release was repeated three times. In this way, the insect's foregut and mouthparts were flushed out by the buffer. The buffer mixture was then pipetted and used for DNA extraction with the DNeasy Tissue kit.

SYBR Green-Based QRT-PCR. RT-PCR was performed in a Rotor Gene 3000 (Corbett Research, Mortlake, Australia) by using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA) (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 U/ml iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, and 20 nM fluorescein) and 4 μl of DNA template in 20-μl reactions. PCR primers, which were originally designed for use with TaqMan QRT-PCR (Schaad et al. 2002), were used for this system: XfF2, 5′ CTC GCC ACC CAT GGT ATT ACT AC 3′ and XfR2 5′ CTG GCG GCA GGC CTA AC 3′. After an

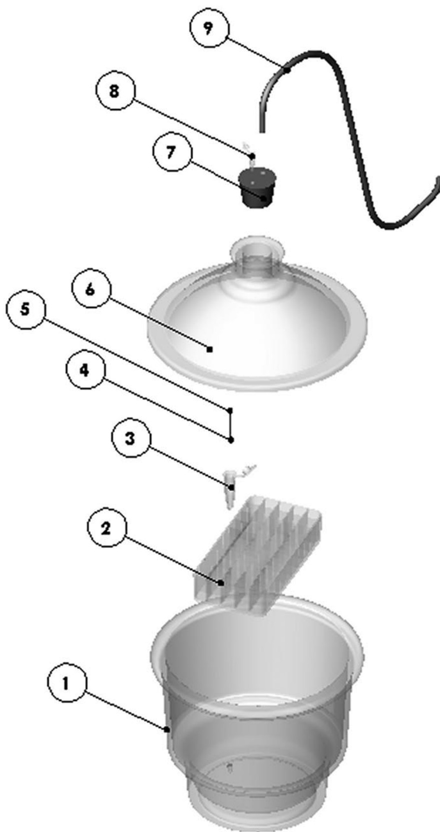

Fig. 2. Breakout view of vacuum extraction setup.

initial denaturing step of 3 min at 95°C, the reactions were cycled 40 times under the following parameters: 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. At the end of the PCR, the temperature was increased from 72° to 99°C at a rate of 1°C/15 s, and the fluorescence was measured every 1°C increase to construct the melting curve. Because SYBR Green fluoresces in the presence of double-stranded DNA (Bates et al. 2001), nontarget amplification increases when primer-dimers form. The target amplicon is created with the XfF2/XfR2 primer at 86.1°C. Therefore, only samples with a melting temperature of 86.1°C were considered positive. This was confirmed by comparing melting temperature-positive samples with agarose gel analysis of the same samples amplified by conventional PCR methods. In all QRT-PCR reactions, a nontemplate control (NTC) was run as a negative control and a known positive sample was run as a positive control.

Comparison of Mortar and Pestle Maceration (MP) to Vacuum Extraction (VE). Vacuum extraction method was compared with a conventional maceration technique. In six replicates, 24 heads per treatment were removed from *H. coagulata* that had been allowed to acquire *X. fastidiosa* (as described above). The heads were either macerated in PBS buffer with a pellet pestle in a disposable 1.5-ml microcentrifuge tube (Kontes Glass Company, Vineland, NJ) or vacuum extracted in PBS buffer, as described above. In both cases, the extraction protocol provided with the DNeasy Tissue kit (QIAGEN) was followed.

Concentrations of *X. fastidiosa* DNA recovered from samples extracted using mortar and pestle maceration were compared with samples processed using vacuum extraction. Estimates of *X. fastidiosa* titer were made with Sample Analysis System Software version 5 (Corbett Research). A χ^2 analysis was used to compare ratios of positive samples between treatments and the mean of relative concentrations between treatments (Preacher 2003).

Comparison of Sample Storage Methods. On two separate dates, *H. coagulata* were collected and maintained on *X. fastidiosa*-positive host plants for 6 d as described previously. Then, the insects were collected and immediately prepared for DNA extraction (n = 24) as described previously or stored at −4°C for 10 d either submerged in mineral oil (n = 24) or not (n = 24). In all treatments DNA extraction and QRT-PCR were conducted as described previously. Comparisons among treatments were made by χ^2 analysis (Preacher 2003).

Detection Capabilities after Insect Trapping. On two separate dates, *H. coagulata* were allowed to acquire *X. fastidiosa* as described previously. All *H. coagulata* were collected and placed by hand on yellow sticky cards (Trécé Inc., Adair, OK). Yellow sticky cards were placed on the western side of the Entomology Building on the University of California, Riverside campus, where it was fully exposed to sunlight in the afternoon. There was no precipitation and average temperature was 24°C with a high of 35°C and a low of 14°C. *H. coagulata* were removed from the traps for DNA extraction at 0, 3, and 6 d after placement. The number of individuals tested was 49 on all days for trial 1 and 30 on all days for trial 2. As described previously, DNA was extracted individually from *H. coagulata*, and QRT-PCR was used for detection. Differences in means were compared between dates by χ^2 analysis (Preacher 2003).

Results

SYBR Green-Based QRT-PCR Detection System. The melting temperature of the 74-bp, 16S rDNA sequence was 86.1°C, whereas the melting temperature of the primer/dimer was only 80.9°C (Fig. 3). Fluorescence was plotted against temperature, resulting in a melting curve. Samples that were considered *X. fastidiosa* positive according to the melting temperature curve were confirmed by visualizing the 74-bp band in an agrose gel.

DNA Extraction. The vacuum extraction technique developed in this study improved the speed and efficiency of extraction. Extraction of DNA by using the more traditional maceration technique with the QIAGEN DNeasy tissue kit averaged 90 min for 24 samples. Approximately 30–40 min of the extraction was preparing for and executing the maceration step of the procedure. By using the vacuum extraction

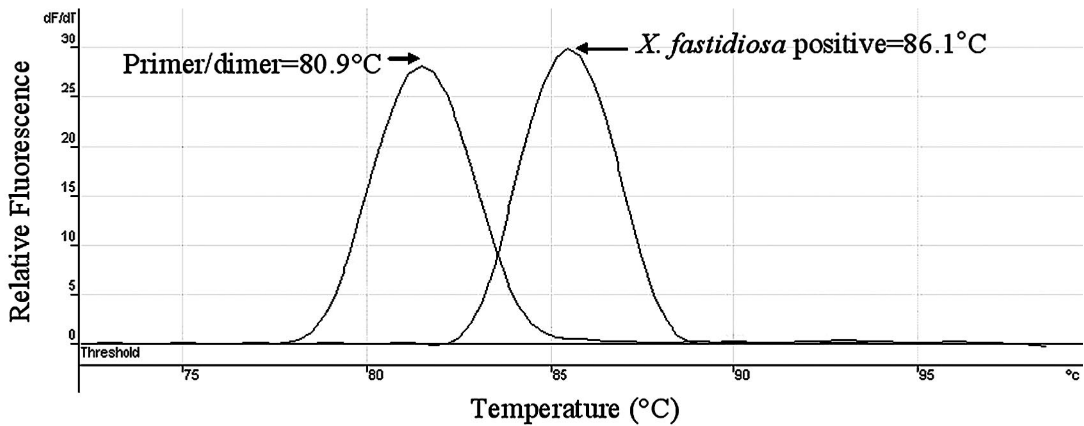

Fig. 3. Real-time recording of melting curve analysis showing the temperature of the *X. fastidiosa* PCR product compared with the primer/dimer amplified in negative samples.

technique, 24 samples were processed in an average of <15 min.

Although the implementation of the vacuum extraction step improved the efficiency of the extraction, it did not improve the ability to detect *X. fastidiosa* in *H. coagulata* heads. However, sensitivity of the extractions was not compromised by implementing this time-saving step. Variation in *X. fastidiosa* acquisition between trials was evident; yet, no statistical differences were detected between maceration-extracted and vacuum-extracted samples in any trial for either the number of positive samples or the relative amounts of *X. fastidiosa* DNA detected in the samples (Table 1). However, in four of six trials mean positives and mean relative detection levels were greater for macerated samples than vacuum-extracted samples (Table 1).

Comparison of Sample Storage Methods. On either collection date, there were no significant differences in mean number of *H. coagulata* testing positive for the presence of *X. fastidiosa* that could be attributed to the method of storage after *H. coagulata* collection (trial 1, $\chi^2 = 1.626$, df = 2, $P = 0.443$; trial 2, $\chi^2 = 2.4$, df = 2, $P = 0.3$) (Table 2).

Table 1. Proportion of *H. coagulata* positive for *X. fastidiosa*, and mean relative fluorescence by using VE and MP sample collection before DNA extraction (n = 24)

Trial	Mean positive[a]		Mean relative fluorescence[b]	
	VE	MP	VE	MP
1	0.458a	0.542a	1.137a	6.299a
2	0.464a	0.789a	1.728a	5.879a
3	1.000a	0.917a	0.112a	0.125a
4	0.917a	0.958a	0.001a	0.003a
5	0.750a	0.917a	0.009a	<0.001a
6	0.917a	0.792a	<0.001a	<0.001a

[a] Means in the same row followed by the same letter were not statistically different ($\chi^2 > 6.6$, df = 1, $P > 0.359$).

[b] Relative fluorescence correlates to cell number. Means in the same row followed by the same letter were not statistically different ($P < 0.01$).

Detection Capabilities after Insect Trapping. Exposure to the elements after capture on sticky cards had little effect on the ability to detect *X. fastidiosa* in *H. coagulata* samples (Table 3). The χ^2 test for goodness-of-fit revealed no statistical differences among means from trial 1 (data taken 0, 3, and 6 d after capture, $\chi^2 = 3.069$, df = 2, $P = 0.216$) or trial 2 (data taken 0, 3, and 6 d after capture, $\chi^2 = 2.845$, df = 2, $P = 0.241$).

Discussion

Previous research indicated a low proportion of *X. fastidiosa*-positive *H. coagulata* in the field where disease spread was rapid (Almeida and Purcell 2003a, Bextine et al. 2004). Traditional techniques to investigate vector-related aspects of field epidemiology are time-consuming due to the number of samples needing to be processed in such an investigation. Our study was conducted to find a means of accelerating the process required to conduct epidemiological studies involving *H. coagulata* spread of *X. fastidiosa,* while maintaining a high degree of detection sensitivity. In our study, we detected no difference in detection rates of the bacterium from infectious *H. coagulata* left on sticky traps in the field for nearly a week. We improved methods of extracting DNA from sites in the sharpshooter where *X. fastidiosa* accumulates in a transmissible form and detected low titers of the pathogen in extracted samples.

Table 2. Comparison of *X. fastidiosa* detection in *H. coagulata* after storage by three methods (n = 24)

Trial	Storage method (n = 24)[a]		
	Directly off plant	−4°C (10 d)	−4°C in mineral oil (10 d)
1	0.875a	0.792a	0.917a
2	0.833a	0.750a	0.917a

[a] Means in the same row followed by the same letter were not statistically different (trial 1, $\chi^2 = 1.626$, df = 2, $P = 0.443$; trial 2, $\chi^2 = 2.4$, df = 2, $P = 0.3$).

Table 3. Proportion of *H. coagulata* positive for *X. fastidiosa* after outdoor exposure on a yellow sticky card

Trial	Mean proportion of *H. coagulata* positive for *X. fastidiosa*[a]		
	day 0	day 3	day 6
1 ($n = 49$)	0.388a	0.429a	0.265a
2 ($n = 30$)	0.533a	0.333a	0.367a

[a] Means in the same row followed by the same letter were not statistically different (trial 1, $\chi^2 = 3.069$, df = 2, $P = 0.216$; trial 2, $\chi^2 = 2.845$, df = 2, $P = 0.241$).

A disadvantage of using a molecular technique such as PCR to detect a pathogen in a host is that detection is based only on the presence of pathogen DNA and does not indicate whether the pathogen was alive at the time of collection. Although other techniques, such as culturing (Almeida and Purcell 2003a), determine the presence of live cells, the ability to detect extremely low titers with such a technique is lower than molecular techniques. The 5–10-d growth period required to observe *X. fastidiosa* colonies on a nutrient agar plate allows time for contaminants to overgrow the plate. Although specialized media are often used for growth, confirmation of bacterial identity is still needed. Although morphological and colony growth characteristics are often used, genetically based identification is more reliable and discriminatory.

The mean number of *H. coagulata* testing positive varied among trials and among experiments. This was most likely due to natural variation in the ability of *H. coagulata* to harbor *X. fastidiosa,* which may be a function of both the insect's age and its exposure to other biotic and abiotic factors that influence the ability of the bacterium to colonize the foregut of *H. coagulata.* Regardless, the point of this study was to develop a detection protocol that could be used despite field conditions. In the six trials where the vacuum procedure was compared with maceration, the mean relative fluorescence values drastically reduced from trial 1 to trial 6, whereas proportions of *H. coagulata* testing positive for the presence of *X. fastidiosa* increased. These experimental replicates were separated by time and were conducted during one season. The variation in these relative fluorescence values could be due to artifacts of the PCR reaction, but the consistent reduction in these values may indicate that *H. coagulata* age may be important in *X. fastidiosa*'s ability to colonize the insect's foregut.

When planning a population survey of infectious *H. coagulata* useful to epidemiological studies, an efficient testing protocol is necessary because of the large numbers of insects that would have to be examined. Additionally, any improvements in the level of *X. fastidiosa* detection would benefit such studies. Through our investigation, we improved the efficiency of *X. fastidiosa* detection by streamlining both DNA extraction and implementing a cost-effective QRT-PCR-based detection system. The vacuum method was simple; requiring only that heads be removed, pinned into position, and covered with extraction buffer. Although time efficiency is the most obvious advantage to using the vacuum extraction method, other advantages also exist that did not impact the data reported here but may affect detection in field samples. First, no insect tissue is homogenized; therefore, it is likely that fewer PCR inhibitors are released to interfere with the PCR reaction and less nontemplate DNA would be extracted. These factors can often hinder detection of pathogen DNA in low concentrations. Second, by flushing the content of the insect's foregut, the search for the presence of *X. fastidiosa* is concentrated in the area of the insect that will most likely contain the organism of interest. QRT-PCR is a sensitive detection technique that detects low concentrations of bacteria in environmental samples (Lockey et al. 1998). The implementation of such a system is well suited for detection of pathogen DNA in an insect vector. By using QRT-PCR, the estimated detection range was between 5 and 50 *X. fastidiosa* cells per insect sample, an improvement of an order of magnitude from detection of 50–500 *X. fastidiosa* cells per insect sample by using traditional PCR as reported previously (Bextine et al. 2004).

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