Comparing Accuracy of *Xylella fastidiosa* Detection Methods in Sharpshooters

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Abstract *Xylella fastidiosa*, a gram-negative bacterium, is spread by a number of insect vectors that causes an array of agricultural diseases including Pierce's Disease in grapevines. The bacterium's recent spread by an invasive vector *Homalodisca coagulata* in southern California has caused a new epidemic of Pierce's Disease throughout California's vineyards. The goal of this study is to measure and compare the accuracy of three different laboratory techniques for the detection of *X. fastidiosa*. These techniques are: polymerase chain reaction-based vacuum extraction from *H. coagulata* mouthparts and foregut where the pathogen is harbored; plant culture using live transmission tests with *H. coagulata* as a vector; and insect head culture of *H. coagulata.* Plant cultures used from live transmission tests demonstrated the highest yield of positive detections of *X. fastidiosa*. Polymerase chain reaction-based vacuum extraction resulted in a low number of positives, and insect head culture was the most inconclusive method with no positives and many contaminations. Although live transmission tests proved to give the highest yield of positives, this process is the most time consuming of the three techniques. Polymerase chain reaction is the fastest method, and will be the most preferred once the errors in the protocol are fixed.

Introduction

California's grape industry is worth over 2.8 billion dollars and is the fourth-leading wine producer in the world (World Institute Website). Products made from grapes include wine, raisin, and table grapes. Over the past century, the emergence of Pierce's disease in grapes has caused an increase in problems for vineyard growers in California. Prominent symptoms of Pierce's disease include leaf scorching, decreased productivity and eventual death of the grapevine (Mircetich *et. al.* 1975). Infections generally occur first on a terminal branch, progressing to adjacent branches; and spreading to the entire plant system within a few years (Mircetich *et. al.* 1975).

Pierce's disease is caused by infection of the pathogen *Xylella fastidiosa*, a bacterium that is vectored by the glassy-winged sharpshooter *Homalodisca coagulata* (Almeida and Purcell 2003). Originally vectored by the blue-green sharpshooter (*Graphocephala atropunctata*), *X. fastidiosa* was first reported in Anaheim, California in the early 1900's (Sorensin and Gill 1996). Now well established, *X. fastidiosa* is transferred to several crops by both *G. atropunctata* and *H. coagulata* causing a wide range of scorch diseases such as alfalfa dwarf, citrus variegated chlorosis, coffee leaf scorch, Pierce's disease caused in grapes, and phony peach disease (Hoddle 2003). Hundreds of strains of *X fastidiosa* have been documented (Hopkins and Purcell 2002).

H. coagulata feeds on the xylem of the grapevine, and during this contact there is a transmission of *X. fastidiosa* (Brlansky *et. al.* 1983). *H. coagulata* harbors this pathogen in the cibarium, an internal structure of the head that lies between the pharynx and the proboscis, and also in the diaphragm (Anonymous Entomology Website). Upon the pathogen's entry into the sharpshooter's jaw, reproduction occurs and the pathogen resides there for the remainder of *H. coagulata's* lifetime (Brlansky *et. al.* 1983). Once *X. fastidiosa* enters the plant's xylem, it multiplies and eventually blocks the movement of water (Brlansky *et. al.* 1983).

With the introduction of *X. fastidiosa* in the past century, California has encountered many problems in its agricultural industry that have yet to be fully solved. Since 1994, over 1,500 wine-grape acres have been damaged by Pierce's disease (Wine Institute Website). With the recent spread of *H. coagulata*, the vector has spread in Southern California and has infected a quarter of Temecula Valley's 3000 vineyard acres, causing millions of dollars in damages (Wine Institute Website). From an accidental introduction in 1989 through imported nursery stock from

the southern United States, *H. coagulata* has now spread to other parts of the state (Wine Institute Website).

Currently, there are three methods of detecting *X. fastidiosa* in the laboratory. These methods are 1) Polymerase chain reaction-based vacuum extraction (Bextine *et. al.* 2004) of *X. fastidiosa* from *H. coagulata* heads, 2) plant culture and 3) insect head culture (Purcell and Finlay 1995; Newman *et. al.* 2004). By doing a comparison study on the accuracy of these different techniques in detecting *X. fastidiosa*, one has the ability to come up with a better approach to lowering populations of the vectors and reducing insecticide use while increasing yields for economic success. By analyzing which procedure is more effective and accurate at determining *X. fastidiosa*, many other studies can be done more effectively such as investigating the viability of vegetation management to decrease insect populations, providing information necessary to develop a treatment threshold for *H. coagulata* populations in areas with widespread *H. coagulata* populations and *X. fastidiosa* infections.

The objective of this study is to measure the accuracy of these three techniques used to detect *X. fastidiosa* in *H. coagulata* in a simultaneous study. I hypothesize PCR-based vacuum extraction will yield the most accurate results compared to live transmission tests and insect head culture.

Materials and Methods

Study Site. This study was conducted at the Purcell Laboratory in the Oxford Tract Greenhouses of the University of California, at Berkeley.

Rearing Insects. Green-house reared *Homalodisca coagulata* were used in this experiment that were field collected from citrus groves in Fresno, CA. Insects were contained in Plexiglas cages with nylon screening. *H. coagulata* were allowed to feed on a variety of host plants including basil, mugworts, and cabernet sauvignon grapes. Plants were changed every three weeks routinely. During this feeding time adult *H. coagulata* laid eggs. Once nymphs hatched from eggs, adults were taken out of the Plexiglas cage for transmission tests. Nymphs then stayed in the cage feeding on different hosts until they grew to be adults. It was not necessary to determine whether *H. coagulata* were pre-infected with *X. fastidiosa* because they fed on infected source plant to acquire the pathogen later on. This cycle is a regular routine at the greenhouse to keep *H. coagulata* on hand year round for transmission experiments (Almeida and Purcell 2003).

Transmission experiments. Originally all transmission experiments were done using the Temecula strain of *X. fastidiosa*. As many results came out negative, the STL strain of *X. fastidiosa* was used for to the remainder of the transmission experiments.

In a 3 x 1.5 x 1.5 ft. Plexiglas cage with nylon screening, approximately 20 greenhouse-reared *H. coagulata* per round were put in with 4 Pierce's disease (PD+) infected cabernet sauvignon grapevines for a 4-day acquisition period (Almeida and Purcell 2003). This grouping of source plants inside the cage was done to minimize source plant variability (Almeida and Purcell 2003). I used an acquisition period of four days to obtain the highest populations transmissions of the pathogen (Almeida and Purcell, 2003). This four day transmission period showed maximized efficiency in a study with blue-green sharpshooter's transmission of *X. fastidiosa* to grapevines (Purcell and Finlay 1979).

Following this feeding acquisition period, surviving *H. coagulata* were placed individually in 5 x 3 inch cylindrical plastic cages with nylon screening for ventilation to confine sharpshooters to non-infected PD- pinot noir seedlings. Six days were allowed for this transmission period when *H. coagulata* fed and transmitted the pathogen to PD- seedlings. During this period seedlings were kept in greenhouse conditions and watered once daily (Purcell and Finlay 1979).

After the 6-day transmission period, remaining *H. coagulata* that were alive were randomly split into two groups. The first group was placed in separate 1.5 mL eppendorf tubes and drowned in acetone for later vacuum-based PCR extraction (Minsavage et al. 1994). The second group was killed immediately, first by freezing the insects to immobilize them, then removing the head with a sterilized razor blade. The removed heads were then ground and cultured, plated on PWG media (Hill and Purcell 1995). Pinot noir seedlings used in the transmission experiments were moved to the greenhouse and cultured after 3 months.

PCR-based vacuum extraction. In order to extract the bacterium *X. fastidiosa* from *H. coagulata* a vacuum extraction method was implemented followed by a commercially available DNeasy tissue kit. The first step in this process was to cut off all the heads of *H. coagulata* with a sterile razor. Care was taken in order to not include the first thoracic segment in the dissection. Heads were transferred to a 96-well ELISA plate and pinned down into separate wells. 180 ?1 of buffer ATL was added to each well and the plate was then transferred to a glass vacuum and the air was taken out. This process was repeated for 5 cycles lasting 10 minutes. Once the ELISA plate was taken out of the vacuum, the pins and heads were then removed. The contents were

transferred to separate 1.6 milliliter microcentrifuge tubes and more buffer was added. 20 ?l of Proteinase K and 200 ?l of buffer AL were added to separate tubes and contents were spun in a vortex immediately. The tubes were then incubated in a heat block at 70 degrees Celsius for 10 minutes. After this incubation period, 200 ?l of 100% ethanol was added to each tube and vortexed immediately. Next, the contents of the tubes were pipetted to a spin column and spun in a centrifuge for 1 minute at 8000 revolutions per minute. The spin columns were then placed over new collection tubes and the flow through (precipitate) in the old tubes were then placed in the hazardous waste bottle in the fume hood. 500 ?l of buffer AW1 was added to the spin columns and spun in a centrifuge for 1 minute at 8000 revolutions per minute. The spin columns were once again placed over new collection tubes and the flow through was discarded. 500 ?l of buffer AW2 was added to the spin columns and spun for 3 minutes at 16,000 revolutions per minute. Spin columns were placed in clean 1.6 milliliter microcentrifuge tubes and the flow through discarded again. 100 ?l of buffer AE was added to the spin columns and incubated for 1 minute. Contents were spun for 1 minute at 8000 revolutions per minute. This step was repeated again, and the final contents in the microcentrifuge tubes were 200 ?l of extracted *Xylella fastidiosa* (Minsavage et al. 1994). Extracted DNA was then run through a polymerase chain reaction with 31' and 33' primers designated for *Xylella fastidiosa* for 4 hours. The magnified DNA was run on a gel in electrophoresis along with 100 kbp marker. Gel electrophoresis is a process that separates macromolecules of different sizes and electric charges. During this process macromolecules are forced to move through the gel when the current is applied. The migration rate through the electric field depends on the size and shape of the macromolecules, relative hydrophobicity of the samples, and ionic strength of the buffer in which the molecules are moving. After all this there is a staining process, where one can view the separated macromolecules in each lane as a series of bands spread from one end of the gel to the other (The Academy for the Advancement of Science and Technology).

Insect culture. The second group of *H. coagulata* that were taken off pinot noir seedling after the transmission period were put into 1.5 mL eppendorf tubes and immobilized by being placed in the freezer for 5 minutes. After *H. coagulata* were immobilized, insects were transferred onto a sterile sheet of Whatman #1 filter paper. The rest of this process was done inside a laminar flow hood with an air filter. The heads were removed using a sterile razor. Once the heads were removed, they were surface-sterilized by being treated in 70% ethanol,

1.5% bleach, and 3 separate water immersions for 1 minute each. Surface-sterilized heads were transferred to a sterile 1.5 mL eppendorf tube containing 100 ?1 of phosphate saline buffer. *H. coagulata* heads were ground for 10 to 20 seconds using a cordless hand grinder with a sterile tip. Once all the heads were ground in buffer, suspensions were plated onto periwinkle wilt (PWG) media and left in sealed plastic bags for 7-10 days inside an incubator set at 28 degrees Celsius (Hill and Purcell 1995).

Plant culture. A minimum of three months after the transmission period was needed before any plant culturing could be done. This wait was implemented in order for symptoms to fully develop from *X. fastidiosa* infection. During this 3 month period plants were kept in greenhouse conditions. The process of plant culturing was similar to the insect culturing technique. Once ready, an arbitrary chosen leaf petiole that appeared symptomatic from the test plant was broken off and surface sterilized, chopped into smaller pieces with a sterile razor and ground in 2mL of phosphate saline buffer with a Polytron homogenizer (Brinkmann Instruments, Inc. Westbury, NY) with a PT-10 generator probe (Hill and Purcell 1993). The grinder was disinfested between samples with successive 10-15 second rinses in sterile water, 95% ethanol, and sterile water again with remaining plant material debris removed with a toothpick. Once samples were ground in buffer, 20 ?1 of each suspension was plated on PWG media and left in sealed plastic bags for 7-10 days inside an incubator set to 28 degrees Celsius (Feil and Purcell 2001).

Solid Media. The solid media that *X. fastidiosa* was plated and grown on is modified Periwinkle-Gelrite (Davis et al. 1983). The media was made by floating 3g of Bovine Serum Albumin (BSA-Sigma #A4503) on top of 15 mL diluted water inside a 50 mL beaker. This beaker was then swirled gently periodically to dissolve the albumin. Next, in a 200 mL beaker, 4g of L-glutamine was dissolved in 100 mL diluted water by heating on a hot plate at low heat. In a separate 2 liter flask, the following compounds were added to 865 mL of diluted water and a stir bar: 4g phytone peptone (BBL #211906), 1g trypticase (tryptone-Fischer# BP 1421-500), 0.4g MgSO4 (Sigma #M1880), 1.2g K2HPO4, 1.0g KH2PO4, 10 mL phenol red stock (Sigma#P4633), 10 mL hemin chloride (Sigma#H5533), and 8g Gelrite gellan gum (Sigma #G1910). The contents of the 2 liter flask were autoclave-sterilized and when cool enough to handle were taken to the laminar flow hood. The dissolved Bovine Serum Albumin and L-glutamine was then sterilized through a vacuum-based filter (Nalgene #121-0020) and added to

the autoclaved mixture. The contents were swirled gently and then poured into Petri-dishes. This recipe made approximately 50 plates (Davis et al. 1983).

Results

After a ten month period of running transmission tests using the vector *H. coagulata* on sick and healthy grapevines, the data collected from a simultaneous study on three separate detection methods are presented in Table 1 and 2. Since contaminations concealed whether XF colonies grew on the solid media, Table 2 takes this into account by making the detection neither positive nor negative.

Table 1: *X. fastidiosa* detection methods in *H. coagulata* glassy-wing sharpshooter heads: comparison of vacuum extraction and PCR; culture on selective media following insect head maceration; and insect transmission of *X. fastidiosa* to healthy grapevines.

Rep.	*X. fastidiosa* Isolate	No. Insects at start	Insect Head Culture X.f. Total	#contaminations	PCR on Head X.f. Total	X.f. Transmission to Plant Total	#contaminations
1	Temecula	16	0 / 8	6	0 / 8	0 / 16	6
2	Temecula	15	0 / 7	6	0 / 8	0 / 15	4
3	Temecula	20	0 / 10	8	2 / 10	0 / 20	7
4	STL	8	0 / 4	1	0 / 4	0 / 8	2
5	STL	11	0 / 5	3	0 / 5	8 / 11	1

Table 2: *X. fastidiosa* detection methods in *H. coagulata* glassy-winged sharpshooter heads after contaminations have been taken out of original detection count.

Rep.	*X. fastidiosa* Isolate	No. Insects at start	Insect Head Culture X.f. Total	PCR on Head X.f. Total	X.f. Transmission to Plant Total
1	Temecula	16	0 / 2	0 / 8	0 / 10
2	Temecula	15	0 / 1	0 / 8	0 / 11
3	Temecula	20	0 / 2	2 / 10	0 / 13
4	STL	8	0 / 3	0 / 4	0 /6
5	STL	11	0 / 2	0 / 5	8 / 10

Insect Head Culture Inconclusive. No positive identifications of the bacterium *X. fastidiosa* were obtained with the insect head culture method. In repetitions 1, 2, and 3 using the Temecula isolate of *X. fastidiosa*, 60-80% of the samples were contaminated. In repetitions 4 and 5, using the STL isolate of *X. fastidiosa*, approximately 44% were contaminated. From these contaminations, conclusions cannot be made whether these were indeed positive or negative for *X. fastidiosa*. Insect head was the most inconclusive method to detect *X. fastidiosa* with no positives and the highest contamination rate of the three techniques (Figure 1). This was the second fastest technique, combining the 10-day acquisition transmission period with a culturing process of about 2 hours. The bacterium then takes about 7-10 days to grow on selective media inside an incubator.

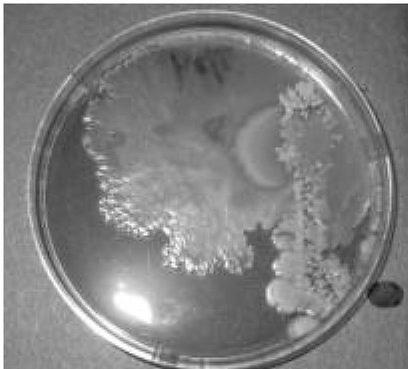

Figure 1: Insect head culture contamination from repetition 3 using Temecula isolate of *X. fastidiosa*. The contamination has spread throughout the whole plate and a positive detection of *X. fastidiosa* cannot be made.

Polymerase Chain Reaction-based Vacuum Extraction Results. Two positive detections of *X. fastidiosa* were found when running the PCR on the extractions from the Temecula isolate of *X.* fastidiosa of repetition 3 (Figure 2). No positive detections were found on the PCR run on repetitions 1 and 2 from the Temecula isolate (Figure 3). There were also no positive detections of *X. fastidiosa* found on the gel run for the PCR of the STL isolate. This was the fastest technique counting the 10-day acquisition period with 2 hours for the DNA vacuum extraction, 4 hours for the PCR to run, and another 2 hours for the gel electrophoresis and staining.

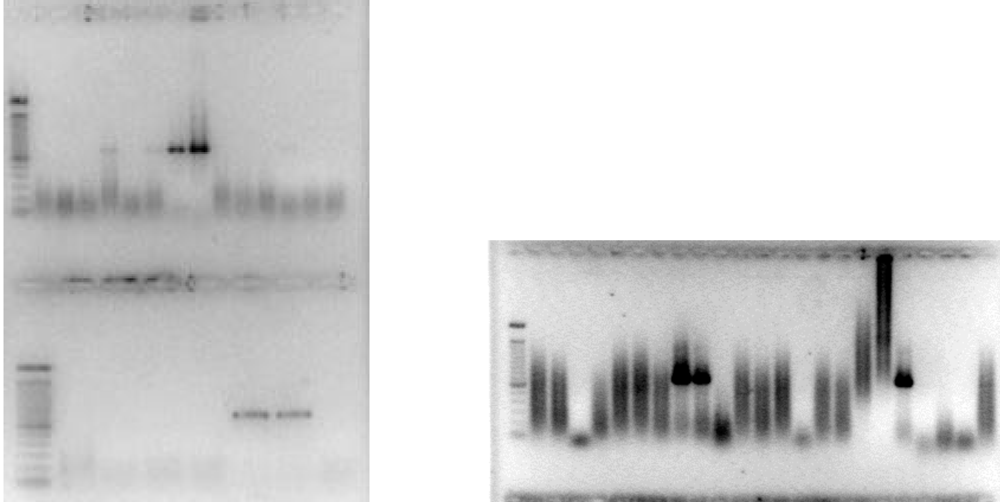

Figure 2a (left): PCR run on gel from repetition 3 that shows two positive detections of *X. fastidiosa* in lanes 12, 30 and positive controls in lanes 7, 8 and 31. Figure 2b (right): PCR on a gel from repetition 1 and 2 that shows only detections were positive controls on lane 8, 9, 18 and 19.

Plant culture Results. After the transmission period with the *H. coagulata* vector**,** plants were stored in the greenhouse for a 5 to 6 month period. A total of 8 positives were detected in repetition 5 with the STL isolate of *X. fastidiosa* (Figure 3). This technique had a greater amount of positives than the other two techniques combined. This was also the most time consuming technique with a long waiting period to allow symptoms to arise in the plants and the 3 to 4 hour culturing process of the petioles. No other positive detections were detected in repetitions 1, 2 and 3 with the Temecula isolate or repetition 4 with the STL isolate. The contamination rate was a lot lower on the plant cultures than the insect head cultures at approximately 30 percent.

Since the plants remained in the greenhouse, the detections that came out contaminated could be re-cultured. Table 3 shows the results for the samples that were cultured again. Four more detections of *X. fastidiosa* were found, 3 in round 1 and 1 in round 3.

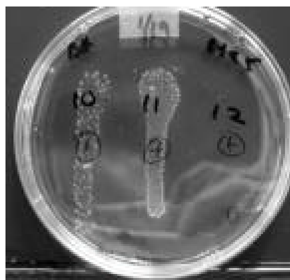

Figure 3: Two positive clusters of *X. fastidiosa.* These colonies were cultured from the transmission plants of repetitions 5 with the STL isolate.

Table 3: *X. fastidiosa* detections after re-testing contaminated samples with plant culture method.

Rep.	Isolate	Contaminations	Re-cultured XF+	Final Total XF+
1	Tem	6	3 / 6	3 / 16
2	Tem	4	0 / 4	0 / 15
3	Tem	7	1 / 7	1 / 20
4	STL	2	0 / 2	0 / 10
5	STL	1	0 / 1	8 / 11

Discussion

The assumption made that vacuum-based polymerase chain reaction extraction of *X. fastidiosa* from *H. coagulata* mouthparts would yield more accurate results than plant and insect bacteria culture was not justified in this study. Instead, I found plant bacteria culture of the grape petioles to have a higher detection rate of *X. fastidiosa* than the other techniques of PCR and insect head bacteria culture. The insect head culture yielded no positive results at all with an averaged 70% contamination ratio. There is not enough conclusive data to run a chi-squared test on the results, so no statistical correlations can be drawn.

I am yet unable to determine whether the uncertain results from the polymerase chain reaction method was due to my laboratory techniques, or if the error was due to unexplainable factors such as the integrity of the kit or equipment. The only two positive results that were concluded from polymerase chain reaction were during the last repetition of the Temecula isolate (round 3). Surprisingly, no positive results were concluded when the isolate of *X. fastidiosa* was changed to the STL variety. From the plant culture data we can infer that during the last repetition of the STL isolate, over 70% of the sharpshooters transferred *X. fastidiosa* to the non-infected test plant. However, no positive results came up during the polymerase chain reaction, meaning there must have been a mistake somewhere along the way because the sharpshooters did have the pathogen in the cibarium mouthpart to transmit it to the plants. There is an advantage using PCR in that no insect tissue is homogenized. When insect tissue is homogenized there is a release of PCR inhibitors that interfere with the PCR reaction (Bextine *et. al.* 2004). Perhaps some faulty technique when removing the head caused an added release of PCR inhibitors that blocked detection of *X. fastidiosa.* A technique that can be implemented for

future studies is diluting the sample to decrease the amount of non-template DNA that has been extracted.

The plant bacteria culture which has shown to be very reliable (Hill and Purcell 1995) was not consistent in this study. There were no positive results until the very last repetition with the STL isolate. Even when there are two positive identifications through the PCR method, there are no bacteria cultures showing on the solid media. The source of this error can most likely be attributed to my faulty sterile techniques or an error in following the protocol. After culturing the samples that were found contaminated, four more positive detections were found.

It is difficult to explain why the insect head culture turned out with no positive outcomes and such high contaminations counts. After discussing this with my advisor Dr. Alexander Purcell, he pointed out a number of different ways contaminations can occur when doing this procedure. One particular source of error occurs during the culturing process after the sharpshooter heads are ground up in a buffer and ready to be plated. It is important that the plates be completely dry with no moisture inside during this time. If the plates have a lot of moisture when the bacteria is plated, all other kinds of bacteria will want to spread around the plate and create an internal environment suitable for a lot of non-*X. fastidiosa* contamination.

When comparing the accuracy of these three techniques, it is also important to remember the time commitment and cost of each protocol. For the laboratory, the PCR technique is the fastest with the least commitment of time, averaging around $5 per sample. The insect-head culture is the second fastest with only the only the cost of making the solid media. The plant culture which has the highest time commitment with the cost of keeping all the plants in the greenhouse space for 4 to 5 months and having them watered daily and sprayed with pesticides weekly. In past studies in my laboratory, the glassy-winged sharpshooters have caused a lot of variable results which have turned out inconclusive in the past. More transmission studies are done with the blue-green sharpshooters because of higher detection rates.

In further study of *H. coagulata* transmission of *X. fastidiosa*, prior to starting any transmission experiments; trying several different dilutions of the extracted DNA from the vacuum technique before running the PCR may help avoid some of the problem with *X. fastidiosa* inhibitors. Also, during the culturing process when plating on solid media, allowing an extended drying period for the agar to fully absorb the suspension might help minimize contaminations of the bacterial culture.

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